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UNIVERSITY OF ALBERTA

REGULATION OF MOTOR UNIT SIZE AND CONTRACTILE PROPERTIES AFTER NERVE INJURIES

BY
VICTOR FRANK RAFUSE

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A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
THE DEPARTMENT OF PHARMACOLOGY

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled,

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PROPERTIES AFTER NERVE INJURIES

submitted by VICTOR FRANK RAFUSE
in partial fulfilment of the requirements for the degree of

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in PHARMACOLOGY (Neuroscience)

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7th December 1992

This work is dedicated to Mom, Dad, Paul, and in loving memory of Beth. Without them I would never have started and certainly not have completed my Thesis.

ABSTRACT

Whether motor nerves can reinnervate more muscle fibers than normal to compensate for incomplete regeneration after complete and partial nerve injuries is not known. This study examined the capacity of motor units (MUs) to enlarge to compensate for reduced MU numbers in partially denervated muscles and in muscles reinnervated following nerve crush injury or after complete nerve transection where the cut nerve was either sutured to its distal stump (N-N suture) or directly to the muscle fascia (N-M suture). These experiments were designed to systematically compare the capacity of "intact" motor axons to form enlarged MUs in partially denervated muscles with regenerating axons. The different paradigms were used to determine whether the type of injury and growth environment affects the capacity of nerves to enlarge their MUs. MUs were isolated in cat medial gastrocnemius muscles, characterized physiologically and histochemically. Indirect measurements of unit innervation ratio (IR) from unit force measurements and direct analysis of the spatial distribution and IR of single glycogen-depleted MUs were performed.

Intact MUs in partially denervated muscles can increase in size in proportion to the reduction in MU number to a limit of 5-8 times normal. All MUs enlarged by the same factor such that the relative difference between small and large MUs remained. Regenerating axons demonstrated the same capacity to form enlarged MUs when the nerve was crushed, or transected and resutured with N-N sutures, but not after N-M suture where nerves are forced to regenerate outside the endoneurial sheath. Thus, regenerating motor nerves are equally able as intact motoneurons to form enlarged MUs, but the nerve sheath is required in order to expand.

Muscle unit fibers in normal and reinnervated muscles occupy discrete territories which are similar in size unless nerves regenerate outside the sheath (N-M suture). The smaller MU territory size after N-M suture indicates that proximal branching of regenerating axons requires the sheath. Thus after N-M suture axons are forced to reinnervate muscle fibers within a smaller MU territory resulting in greater clumping of muscle unit fibers. The progressively higher clumping of muscle unit fibers with decreasing MU number is paralleled by fiber type clumping showing that type-grouping is a better indicator of reinnervated MU numbers rather than simple regeneration.

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μV = microvolts

mm = Millimeters

mN = Millinewtons

MU = Motor unit

N = Newtons

nA = nanoamperes

N-CAM = Neural cell adhesion molecule

N-M = Nerve-muscle suture

N-N = Nerve-nerve suture

PAS = Periodic acid-Schiff stain

PD = Partial denervation

p_i = Reletive frequency of muscle fiber types

q_i = Relative frequency of motor unit types

RO = Regression coefficient

S = Slow motor unit

SD = Standard Deviation

SE = Standard error

sec = Second

SF = Specific force

SO = Slow-oxidative muscle fiber

TA = Tibialis anterior muscle

VR = Ventral root

LIST OF ABBREVIATIONS

ATPase = adenosine triphosphatase

CAP = compound action potential

CSA = cross-sectional area

EMG = Electromyogram

FDL = Flexor digitorum longus muscle

FF = Fast-fatigable motor unit

FG = Fast-glycolytic muscle fiber

FI = Fast-fatigue intermediate motor unit

FOG = fast-oxidative glycolytic muscle fiber

FR = Fast-fatigue resistant motor unit

g = Gram

1/2 FT = Half fall time

Hz = Hertz

IP = Intraperitoneal

IR = Innervation ratio

kg = Kilogram

LG = Lateral gastrocnemius muscle

M = Total number of muscle fibers in muscle

m = Slope of regression line

mg = milligram

MG = Medial gastrocnemius muscle

μm^2 = square micometers

1. GENERAL INTRODUCTION

In 1930, Eccles and Sherrington (1930) suggested that the wide range in diameter and conduction velocity of motor axons was associated with a wide range in innervation ratios (IRs; number of muscle fibers innervated by a single motoneuron) among motor units (MUs). In the mid 1960's, Henneman and colleagues isolated single motor units for the first time in the cat soleus (McPhedran et al. 1965) and medial gastrocnemius (MG) muscles (Wuerker et al. 1965). In these experiments they showed firstly that MU contraction times, unit force, and conduction velocities vary over a wide range. A second finding of equally great importance was that axon conduction velocity was directly related to the force of the MU. Using conduction velocity as an electrophysiological measure of axon diameter (Hursh 1939), they concluded that in a homogeneous muscle such as the cat soleus, where the muscle fibers are relatively uniform in size, the number of fibers innervated by a single motoneuron (IR) is directly related to its size (Henneman and Olson 1965). That is, larger motoneurons innervate more muscle fibers than smaller motoneurons. Extrapolating from Clarke's (1931) average IR of 120 fibers in the cat soleus and the range in unit tetanic force (3.2 - 40.4 g) they estimated that the smallest MU contains 39 fibers while the largest MU 491 muscle fibers (McPhedran et al. 1965). Since the largest and mean unit force in the cat MG muscle is approximately 3 times that of soleus, they estimated that the largest MG unit contains 1,473 fibers while the mean MU size was 540 fibers (Wuerker et al. 1965). However, since the MG muscle contains 3 muscle fiber types that vary in size the authors warned that such estimates in the MG must be interpreted with caution.

1.1 Determinants of MU force.

Unit force normally varies dramatically between different muscles and between different unit types within a given muscle. For example, unit force in the slow contracting cat soleus varies over a 13- fold range (McPhedran et al. 1965), whereas unit force in the larger heterogeneous cat MG muscle varies over a 100- fold range (Burke et al. 1973). As described by Burke (Burke 1981), unit force (F) is the product of three factors:

$$F = N \times CSA \times SF$$

where N in the number of muscle fibers innervated by a single motoneuron (innervation ratio; IR), CSA is the mean cross-sectional area of the muscle unit fibers, and SF is the intrinsic contractility of the muscle unit fibers (ie. specific force or the force generated per unit CSA). The normal variation in unit force within a single muscle, and between different muscles, can be therefore be attributed to differences in any one or all three of these factors.

In a series of studies in the early 1970's, Burke and colleagues (Burke and Tsairis 1973; Burke et al. 1973) further classified MUs according to their physiological properties, and indirectly estimated the relative contribution of each of the three factors in determining the normal wide range in unit force. Using isometric contractions MUs were classified mainly according to the mechanical properties of their associated muscle fibers. Slow and fast contracting MUs were identified according to the absence or presence of sag during an unfused tetanus, respectively. Slow units were all resistant to fatigue and were slow contracting. Fast units, on the other hand, had faster contraction times and showed variably resistance to fatigue. Fast units were, therefore, subsequently divided into fast fatigue resistant (FR), fast fatigue intermediate (FI), and fast-fatigable (FF) according to their susceptibility to fatigue during a two minute fatigue test.

Using the glycogen-depletion technique developed by Edstrom and Kugelberg (1968), Burke repetitively stimulated single MUs to depleted their muscle fibers of their glycogen stores. By comparing serial whole muscle cross-sections stained for periodic acid Shift (PAS, Pearse 1960), and for myosin ATPase activity, according to the methods of Brooke and Kaiser (1970), they first confirmed the observations of Edstrom and Kugelberg (1968) that all muscle fibers belonging to the same MU were of the same histochemical type and second that the staining profile of each physiologically identified MU type was different. FF units were found to contain type IIB fibers, FR units type IIA fibers, and S units type I fibers.

On the basis of the correspondence of S, FR and FF units with type I, IIA and muscle fibers, Burke et al. (1973) estimated IR indirectly by obtaining relative frequencies of motoneurons from MU types and muscle fibers from muscle fiber types. The average IR for each MU type was estimated by the following equation

$$\text{Mean IR} = (M/N) \times (p_i/q_i)$$

where M equals the total number of muscle fibers in the whole muscle, N is the total number of motoneurons innervating the muscle, and p_i and q_i are the relative frequency of the different muscle fiber and MU types. Using this indirect measure of IR, Burke and Tsairis (1973) estimated that the mean IR of S, FR, and FF units were 78, 78, and 124 fibers, respectively. Using estimated IRs and mean CSAs of different muscle fiber types, SF for each MU type was calculated from equation 1. Since differences in the mean CSA of the different fiber types and variation in IR could not account for the large difference in mean unit force between S and FF units, they concluded that variation in unit force was primarily due to differences in SF of the 3 muscle fiber types and not IR as suggested by Henneman.

The validity of the indirect measurements depends on how representative the sample of muscle unit fiber and MU types is of the whole muscle populations. Since several muscles are highly regionalized with respect to different fiber types along the muscles transverse axis (Dennny-Brown 1929; Pullen 1987), and longitudinal axis (English and Letbetter 1982), accurate samples of the true proportion of muscle fibers is difficult. In addition, the proportion of F units may be overestimated because large motoneurons are more easily penetrated than S units during intracellular stimulation (Henneman 1980). Sampling errors in both the proportion of different muscle fiber and MU types can lead to an underestimate of the mean IR of different MUs and an overestimate in the contribution of SF to the range in unit force (see also Discussion in Bodine et al 1987; Stein et al. 1990; Tötösy de Zepetnek et al. 1992).

For these reasons 4 recent studies, using the technique of glycogen-depletion, have reexamined the question as to what extent the normal range in unit force is determined by differences in IR, mean CSA, and SF (Bodine et al. 1987; Chamberlain and Lewis 1989; Kanda and Hashizume 1992; Tötösy de Zepetnek et al. 1992). In two studies the tibialis anterior (TA) muscle was chosen (cat: Bodine et al. 1987; rat: Tötösy de Zepetnek et al. 1992) because the muscle is relatively small, the muscle fibers are long and the pinnation angle small, thereby ensuring that a single muscle cross-section contains all muscle fibers from a single MU (Sacks and Roy 1982). The results from both studies show that IR is a major factor determining the normal wide range in unit force. Differences in mean muscle unit fiber CSA was also a significant determinant of unit force whereas SF was only slightly modulated between different MU types. The results from Chamberlain and Lewis (1989) in the rat soleus, and Kanda and Hashizume (1992) in the rat MG, are consistent with these conclusions.

Following reinnervation of the rat TA muscle, after section and resuture of the common peroneal nerve, the normal range in unit force was reestablished and IR varied positively with unit tetanic force as in normal muscles (Tötösy de Zepetnek et al. 1992). In fact the contribution of IR was even greater in reinnervated muscles as compared to normal due to the smaller differences in mean muscle fiber CSA between different MUs (see below).

Thus, differences in the extent of branching of motor nerves is an important factor determining the wide range in unit tetanic force in normal and reinnervated muscles. The finding that electrophysiological measures of axon size is well correlated with unit tetanic force in normal (Appelberg and Emonet-Denand 1967; Wuerker et al. 1965, Gordon and Stein 1982a; Gordon et al 1988) and reinnervated muscles (Gordon and Stein 1982a; Gordon et al. 1986;1988) and that IR varies as a function of unit force provides strong evidence that motor axons branch in a size-dependent manner, as originally suggested by Henneman, during both development and regeneration.

However, there are several lines of evidence that suggest that the size of the reinnervated MU may not be solely governed by the diameter of the motor axon, but rather the size may be dependent on the relative activity of the motoneuron or the growth environment of the regenerating axons. Several studies on reinnervated MUs have shown that small MUs increase in size more than larger MUs (Desypris and Parry 1990; Foehring et al. 1986; Gordon and Stein 1982b; Lewis et al. 1982; Tötösy de Zepetnek et al. 1992). In addition, several studies on MU properties in self- and cross-reinnervated muscles have shown that unit force is either similar to, or less than normal, even when fewer MUs reinnervate the muscle than normal (Chan et al. 1982; Foehring et al. 1986; Foehring et al. 1987; Gordon and Stein 1982ab; Gordon et al. 1986;19[illegible] however see Bagust and Lewis 1974; Desypris and Parry; 1990; Tötösy de Zepetnek et al. 1992). In a series of chronic and acute recordings of reinnervating MUs, Gordon and Stein (1982ab) showed that after section and resuture of the cat MG nerve to its distal stump (nerve-nerve suture; N-N), whole muscle force recovered to its preoperative values. In contrast, when the MG nerve was sectioned and sutured directly to the muscle fascia (nerve-muscle suture; N-M), 50% of the muscles only recovered 15-50% of their preoperative force. Since the mean unit tetanic force returned to normal values in both conditions the smaller recovery of force in reinnervated muscles after N-M suture was due to fewer motor axons growing back to the muscle to make functional nerve-muscle connections. These results imply that either regenerated MUs do not have the capacity to enlarge in size to innervate more muscle fibers than normal or that the growth environment is less conducive for branching after N-M suture.

Adult motoneurons clearly have the capacity to enlarge in size and innervate a greater number of muscle fibers than normal by extending collateral nodal, terminal and/or ultraterminal sprouts to supply denervated muscle fibers following partial denervation (Brown and Ironton 1978; Edds 1950; Thompson and Jansen 1977). Several studies in rat (Fisher et al. 1989; Gorio et al. 1983; Thompson and Jansen 1977;), mouse (Brown and Ironton 1978; Fladby and Jansen 1988) and humans (Yang et al. 1990) have shown that MUs can increase in size to a limit of 3-5 times that of normal. Whether all MU types increase in size by the same extent is not known. The observation that intact motor axons enlarge to the same size as that found in the neonate during the period of polyneuronal innervation (Brown et al. 1976; Redfern 1970) has led some investigators to suggest that this limit represents the upper capacity of MUs enlargement (Thompson and Jansen 1977). Several possible factors have been suggested to explain this limited sprouting capacity including a limited availability of trophic factors from the muscle (Brown 1984) or a limit in the metabolic capacity of motoneurons to supply an increased number of muscle fibers (Rochel and Robbins 1988; Tissenbaum and Parry 1991). Alternatively, the perimysial sheaths of connective tissue may act as barriers that restrict the growth and elongation of sprouts (Kugelberg et al. 1970).

1.2 Spatial distribution of muscle unit fibers

A major breakthrough in our understanding of the anatomical properties of single MUs came with the development of the glycogen-depletion technique of Edstrom and Kugelberg (1968). Using this technique they showed two major principles in the anatomical organization of single MUs. First, all muscle fibers within a single MU are of the same histochemical type and second, that the muscle fibers are "scattered" in a mosaic pattern within a given region of the muscle. This scattered distribution of muscle fibers indicates that any given area of the muscle is occupied by several muscle fibers belong to different MUs with overlapping territories. In addition, the MU territories do not span throughout the entire muscle but rather are located in discrete regions of the muscle cross-section (Bodine-Fowler et al. 1990; Edstrom and Kugelberg 1968; English and Weeks 1984; Tötösy de Zepetnek et al. 1992).

A striking observation following partial denervation (Brandstater and Lambert 1973; Kugelberg et al. 1970), or reinnervation of murine muscles (Kugelberg et al. 1970; Tötösy de Zepetnek et al. 1992) is the fibers from single MUs are no longer scattered in a mosaic pattern, but rather are tightly aggregated in clumps. In addition, the size of the MU territory is dramatically decreased following reinnervation (Kugelberg et al. 1970; Tötösy de Zepetnek 1990). These observations indicate, firstly, that the regenerating axons do not reinnervate their original muscle fibers and, secondly, that the branching pattern of the motoneuron is dramatically different from normal. What factors determine the extent of branching of regenerating motor axons are not known.

Impressed by the observation that the boundaries of the MU territory in partially denervated, and reinnervated muscles coincided with the fascicles of the muscles, Kugelberg et al (1970) proposed that the perimysial sheaths of connective tissue may act as barriers preventing sprouts from extending beyond the fascicles. As a result, MUs enlargement is primarily due to innervation of muscle fibers within the MU territory. Reinnervated MUs typically have smaller territories compared to normal (Kugelberg et al. 1970). If regenerating motor axons primarily reinnervate muscle fibers within the unit territory, a smaller territory would reduce the number of available fibers for the axon to innervate which in turn would limit the size of the regenerated MU. If the number of regenerating nerves is experimentally decreased one would expect, from this hypothesis, that the extent of clumping of muscle unit fibers within the territory would increase as the MU was forced to enlarge to compensate for the reduced MU number. Examination of the spatial distribution patterns of glycogen-depleted muscle units, under conditions where the MU number is reduced, may provide some important insights into what factors regulate branching which ultimately determines MU size.

1.3 MU plasticity

In 1960, Buller and coworkers (Buller et al. 1960) published what is now considered classical experiments which showed that cross-reinnervation of the fast-twitch digitorum longus (FDL) muscle by the motor nerve to the slow-twitch soleus muscle, or vice versa, changed the speed of contraction of the reinnervated muscle in the direction according to the foreign nerve's original muscle. These results were of great importance since they were the first to show that the muscle contractile properties are regulated by the innervating motoneuron.

After complete nerve transection regenerating nerves do not reinnervate their original muscle fibers even when they are sutured to their original muscles (Gordon et al. 1988; Kugelberg et al. 1970; Tötösy de Zepetnek et al. 1992; Warszawski et al. 1975). The finding that after long-term reinnervation, the muscle fibers within a single reinnervated MU all have the same phenotype supports the suggestion by Buller and colleagues that the innervating motor nerve determines the muscle fiber histochemical profile.

Following self-reinnervation the normal relationships between axon potential amplitude, twitch contraction time, and unit tetanic force is initially lost when the muscles were first reinnervated, but return with time (Gordon and Stein 1982ab). Since completely transected nerves do not reinnervate their former muscle fibers the reestablishment of these normal size relationships supports the suggestion by Buller and colleagues that the innervating motor nerve determines the muscle fiber contractile properties.

However there are several lines of evidence to suggest that conversion of muscle fiber phenotypes by regenerating motor axons is incomplete. Although the formerly fast-twitch FDL muscle was converted to slow in the original cross-reinnervation study of Buller et al. (1960), the contractile speed of the soleus was not completely converted to values comparable with MUs in normal FDL muscles. Several studies have shown that MUs in cross-reinnervated soleus muscles retain their high endurance and oxidative capacities despite the fact that they are reinnervated by nerves formerly supplying fast-twitch muscles (Chan et al. 19882; Dum et al. 1985; Foehring et al. 1987; Gillespie et al. 1987). In addition, fast MUs in cross-reinnervated soleus muscles do not always produce a sag response during an unfused tetanus (Dum et al. 1985) and there is consistently a greater number of FI units in reinnervated cat MG muscles (Gordon et al. 1986;1988) suggesting that there is incomplete regulation of the contractile mechanisms and conversion of oxidative/glycolytic enzymes by the innervating motoneuron.

There are also examples of incomplete regulation of muscle fiber properties by reinnervating motoneurons. Muscle fibers in reinnervated MUs express both fast and slow myosin heavy chains despite the fact that all fibers were of the same histochemical type (Gauthier et al. 1983). Differences in muscle fiber size within a given type becomes less evident after reinnervation (Gordon et al 1988) with increasing overlap in the range of sizes. In addition, the range in muscle fiber CSA within a single MU is also significantly greater than normal implying incomplete regulation of fiber size by the reinnervating motoneuron (Tötösy de Zepetnek et al. 1992).

Taken together, these results suggest that the innervating nerve has a strong influence on rematching nerve and muscle properties in reinnervated MUs. However, the apparent incomplete conversion of contractile, biochemical, and histochemical muscle fiber properties argues that factors other than the nerve are important in regulating muscle fiber properties.

1.4 Summary of objectives

The specific objectives of this study were:

1) to determine the capacity of motoneurons to increase their MU size by collateral sprouting in partially denervated cat MG, lateral gastrocnemius (LG), and soleus muscles,

2) to determine whether the increase in mean unit force in partially denervated muscles is a function of the number of reinnervated MUs,

3) to determine whether all MUs increase in size by the same extent and whether the increase is MU size is a function of the size the motoneuron,

4) to determine whether regenerating motor axons have the same capacity to increase in size as intact axons in partially denervated muscles,

5) to compare the capacity of reinnervated MUs to enlarge under conditions where the nerve has either been crushed, or completely transected and resutured to the distal nerve stump (N-N suture) or directly to the muscle fascia (N-M suture). To force MUs to increase in size, the number of regenerating nerves has also been experimentally reduced,

6) to compare the spatial distribution of muscle unit fibers in normal and reinnervated muscles to indirectly analyze the pattern of motor axon branching,

7) to determine to what extent the regenerating motoneurons reestablish normal MU contractile properties and to determine whether the normal size relationships between axon size and unit force returns,

8) to reexamine the contribution of IR, CSA, and SF in determining the normal wide range in MG unit force using a model of chronic electrical stimulation.

Motoneurons innervating partially denervated cat triceps surae muscles increased their MU size (IR) in direct proportion to the extent of partial denervation to a limit of 5-6 times that of normal. All MUs increased in size by the same factor such that the normal size relationship between axon diameter and unit tetanic force was maintained indicating that MUs increased their IR as a direct function of motor axon size (see Chapter 2).

Regenerating motor axons have the same capacity to enlarge the size of their MUs as motoneurons in partially denervated muscles provided that the axons elongate within the distal endoneurial sheaths (ie. following nerve crush injury or after complete nerve transection and repair with N-N sutures). Regenerating axons cannot enlarge their MU size (ie. increase IR) beyond normal values if they are forced to reinnervate denervated muscle fibers by elongating outside the nerve sheaths (ie. N-M suture) (see Chapter 3).

Spatial analysis of the distribution of muscle unit fibers from a single glycogen depleted MU indicates that regenerating motor axons reestablish the normal compartmentalization of MUs in the cat MG muscle. Motor axons regenerating outside the endoneurial sheaths make fewer proximal branches compared to regenerating axons elongating within sheaths with the result that the MU territory is smaller and the extent of muscle unit fiber clumping within the territory greater. The smaller MU territory ultimately reduces the capacity for regenerating motor axons to from enlarged MUs following N-M suture since there are simply fewer denervated fibers within the MU territory to reinnervate (see Chapter 4).

Regenerated motoneurons reestablished the normal range in MU contractile properties and reestablished the normal relationships between axon size and contractile speed with unit tetanic force. These results are consistent with the idea that motoneuronal properties determine the size of the MU and the contractile properties of the muscle fibers. However, the observed increase in FI units and the wider range in muscle fiber CSA within single MUs in reinnervated muscles indicates that neuronal control of muscle fiber properties is incomplete (see Chapter 5).

To determine to what extent different unit IRs contribute to the wide range in unit tetanic force the cat MG muscle was chronically stimulated at a low frequency to eliminate muscle fiber CSA and SF as contributing factors to the range in unit force. After 142 days of stimulation both the mean muscle unit fiber CSA between different MUs and the SF of all fibers were estimated to be the same. Consequently, the remaining 35- fold range in unit tetanic force was attributed to be solely due to a 35-fold range in unit IRs. IR is therefore the major determinant controlling the normal 100-fold range in unit tetanic force in the cat MG muscle (see Chapter 6).

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2. PROPORTIONAL ENLARGEMENT OF MOTOR UNITS AFTER PARTIAL DENERVATION OF CAT TRICEPS SURAE MUSCLES

2.1 INTRODUCTION

Each motoneuron supplies hundreds and even thousands of muscle fibers and may sprout to supply many more after partial denervation (PD) of a muscle or as a result of clinical nerve trauma and/or disease (reviewed by Brown et al. 1981; Wernig and Herrera 1986). However, factors that control and limit motor unit (MU) size, namely the numbers of muscle fibers per motoneuron (innervation ratio, IR), are not well understood.

During early mammalian development, when each muscle fiber shares innervation with two to five other motoneurons (polyneuronal innervation, Bagust et al. 1973; Brown et al. 1976; O'Brien et al. 1978; Redfern 1970), MUs initially include up to 5 times as many muscle fibers as in the adult. The excess terminals are withdrawn during postnatal development to establish the single innervation of the mature muscle fibers. In so doing, immature MUs are reduced in size by a factor of 2-5. Adult motoneurons nevertheless maintain the capacity to increase the number of their branches and supply more muscle fibers by extending nodal, terminal, and/or ultraterminal sprouts to reinnervate denervated muscle fibers (Brown and Ironton 1978; Edds 1950; Rochel and Robbins 1988). However, the extent to which adult MU size can increase remains controversial. Increases of 3-5 times in partially denervated muscles in mouse (Brown and Ironton

1978; Fladby and Jansen 1988), rat (Fisher et al. 1989; Gorio et al. 1983; Thompson and Jansen 1977) and humans (Yang et al. 1990) have been reported but there are examples of increases of as much as 10 to 20 times the normal size in rabbit and cat muscles, respectively (van Harreveld 1945; Luff et al. 1988). Wide differences in the methods used to measure MU size and muscle functional recovery and in the number of MUs sampled are all factors that could contribute to this variability.

Normally, MU force is well correlated with nerve fiber diameter as measured electrophysiologically (Bagust 1974; Emonet-Denand et al. 1988; Gordon and Stein 1982a; McPhedran et al. 1965; Wuerker et al. 1965; however, see also Stephens and Stuart 1975; Zajac and Faden 1985). Because IR is an important determinant of unit force, it follows that MU size (measured either as MU force or IR) varies as a function of the size of the motor nerve (Stein et al. 1990; Tötösy de Zepetnek et al. 1992). Since unit force, IR and axonal size also covary in reinnervated muscles, regenerating nerves appear to reestablish the size relationships by branching in proportion to the size of the motor nerve (Gordon and Stein 1982a; Tötösy de Zepetnek et al. 1992). The logical extension of these findings is to ask whether the same size relationships are reestablished for enlarged MUs in partially denervated muscles.

A related question is whether the level of activity of motoneurons influences their ability to enlarge their MU size by sprouting, because the smallest and slowest MUs are recruited into activity before the larger and faster MUs, in accordance with the orderly recruitment of MUs according to size (Milner-Brown et al. 1973; Dengler et al. 1988). Several investigators have noted that after self- or cross-reinnervation of denervated muscles, the slow MUs are frequently larger than normal (Foehring et al. 1986; Gordon and Stein 1982a; Gordon et al. 1988; Lewis et al. 1982; Tötösy de Zepetnek et al. 1992). Studies in which some of the regenerating nerves were inactivated by tetrodotoxin blockade indicate that inactive motoneurons are at a competitive disadvantage and form smaller MUs than those that remain active (Ribchester and Taxt 1983). However, the findings that force and IR increase as a function of motor nerve size after reinnervation (Gordon and Stein 1982a; Gordon et al. 1986b; Tötösy de Zepetnek et al. 1992) would suggest that MU size increases inversely, rather than directly, with the level of activity of the MUs in reinnervated as well as in normal muscles. Recent studies of the effects of functional overload (synergist ablation) on the capacity of rat plantaris muscles to recover from PD showed that increased neuromuscular activity did not increase sprouting capacity, although synaptic efficacy was compromised (Michel and Gardiner 1989). The present study investigates the influence of neuromuscular activity on sprouting capacity by determining whether there is a differential increase in unit force for MUs that differ widely in their size and recruitment order.

We have recorded MU force and axon potential amplitude, as measures of MU size and axon size, respectively, in the triceps surae muscles of the cat, to systematically study the response of a MU population to PD. By obtaining a large representative sample of MUs and using methods to quantify the number of remaining MUs after partial lesion, we can assess firstly, whether all remaining MUs contribute equally in supplying denervated muscle fibers, and secondly, whether MU size increases as an inverse function of the numbers of remaining MUs. Thirdly, we have compared MU size in muscles in which PD was carried out in the adult or during neonatal life. This comparison was made to examine if final MU size is the same whether the MUs are within the normal mature size range or initially larger than normal, as is the case in adult and neonatal muscles, respectively. Finally, the stability of the enlarged MUs was examined by permitting regeneration of the cut nerves to determine whether regenerating axons can displace sprouted axons from their terminals.

Results have been published in abstract form (Gordon and Orozco 1987; Rafuse et al. 1990).

2.2 METHODS

2.2.1 Preparation and surgery

A total of 30 cats were used in this study including 8 control cats, sex and age matched for the final weight (2.5 - 5.1 kg) of the 22 experimental animals. In the experimental group, 15 adult cats (2.2 - 3.6 kg) and 7 three to four week old kittens (0.3 - 0.6 kg) were anesthetized with pentobarbital sodium (45.0 mg/kg) for partial denervation of the triceps surae muscles of one limb. Under strict aseptic conditions, either the right L_7 or S_1 spinal root was cut; the L_7 spinal root in 4 animals, the S_1 root in 17 animals and both L_7 and S_1 roots in 1 animal. No precautions were taken to prevent regeneration of the cut axons. The cats were housed in large cages that permitted free movement and playful activities for 2.5 - 18 months before a final acute experiment was performed. Thirteen experimental animals were used to study partially denervated medial gastrocnemius (MG) muscles (10 operated as adults, 3 as kittens) and 9 animals were used to study lateral gastrocnemius (LG) and soleus muscles (5 operated as adults, 4 as kittens). Because not all physiological and histological measurements were made on each cat, the number of animals used is shown in the table and figure legends.

In the final acute experiment, the cats were anesthetized with pentobarbital sodium, the brachial vein was cannulated for additional injections of anesthetic (1.0 % of the initial dose) to maintain deep anesthesia, and the cats were intubated with a tracheal cannula for artificial ventilation if required. A laminectomy was performed to expose the L_7 and S_1 ventral roots (VRs). All muscles other than the MG, LG, and soleus muscles were denervated by section of all branches of the sciatic nerve (Sc) including those that supply the hip and hamstring muscles and the common peroneal (CP) and tibial (Tib) nerves which supply the lower limb muscles (Fig. 2.1A). The MG and lateral gastrocnemius-soleus (LGS) nerves were left intact. The MG, LG and soleus muscles were exposed in both hindlimbs and, as far as possible, freed from each other and connective tissue. Care was taken to keep the blood supply intact. The muscle tendons were securely fastened with silk thread (no. 5) and tied to a 10-kg force transducer to measure total muscle force and subsequently to a 2-kg transducer for single MU force measurements (Grass Instruments, models FT10 and FT03, respectively). In all cases a small piece of calcaneus bone was left in continuity with the tendon to prevent slippage of the thread during maximal contractions.

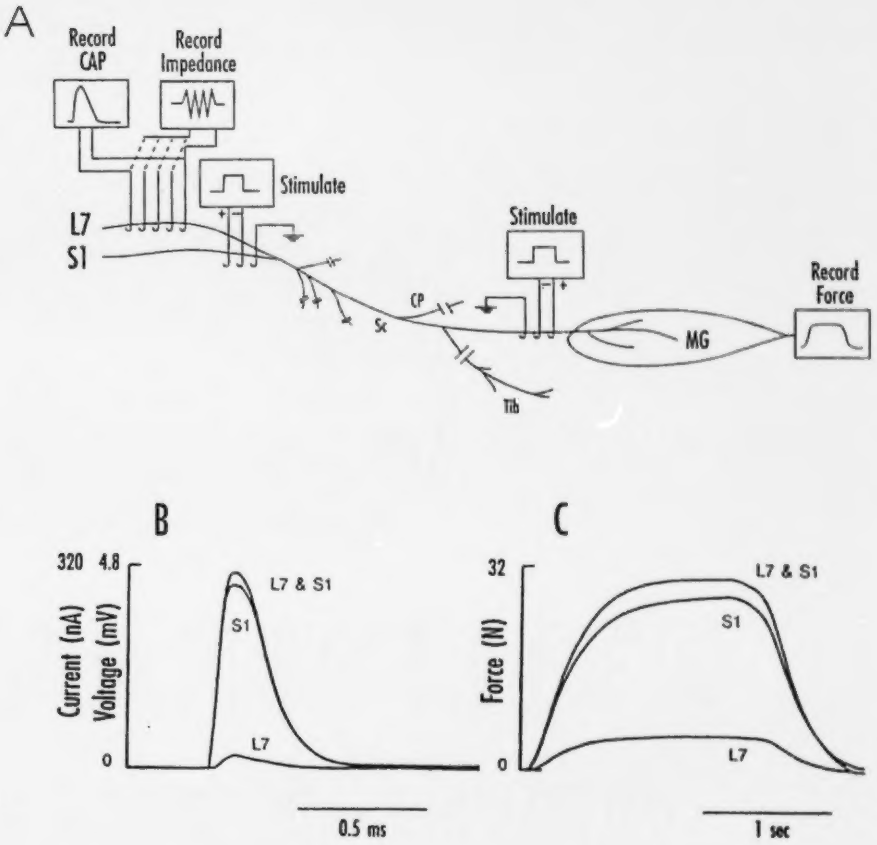
The animals were placed prone on a heating pad, mounted on a stereotaxic frame, and secured with clamps at the hips, knees, and ankles. The skin surrounding the laminectomy and hindlimb muscles on both legs was drawn up to form three pools, which were filled with medicinal grade mineral oil maintained at 34°C. Rectal temperature was maintained at 37°C.

2.2.2 Quantification of MU number

There is bilateral symmetry in the relative contribution of L_7 and S_1 VRs to the motor innervation of MG, LG and soleus muscles (Buller and Pope 1977; Gordon et al. 1986a; Hoffer et al. 1979; van Harreveld 1945). Therefore the relative contribution of each VR to the innervation of a muscle on one side provides a reliable estimate of its contribution on the contralateral side. We therefore determined the contributions of S_1 and L_7 VRs to the muscles in the unoperated hindlimb to obtain a reasonable estimate of the number of motor nerves that had been cut (usually S_1) or left intact (usually L_7) on the contralateral operated side. The relative VR contributions to the three triceps surae muscles were determined by measuring 1) the charge contribution of each of the two VRs in response to stimulation of the MG and LGS nerves (Fig. 2.1B) and 2) the muscle force elicited by tetanic stimulation (100 Hz) of each VR separately (Fig. 2.1C). Each root was cut at the exit point from the spinal cord, and a five-electrode array was used to measure the impedance of the cut root as described in detail by Hoffer et al. (1979). Monophasic compound action potentials (CAPs) were elicited by stimulation of MG or LGS nerves and recorded using the outermost electrodes of the array. The CAP was normalized by the impedance (kΩ) to derive values of current (nA) and, by integration, charge (pC). Units of charge were used because charge can be directly compared between VRs of different sizes. The charge contribution of each VR reflects the number of fibers contributing to the CAP and their cross-sectional area (CSA). The mean values (± S.E.) of axon potential amplitude and conduction velocity of 182 normal MUs isolated from the L_7 and S_1 VRs are not significantly different (compare 23.9 ± 0.5 and 22.8 ± 1.3 uV; 94.9 ± 1.2 and 93.8 ± 2.1 m/s, for 143 and 39 units in L_7 and S_1 VRs, respectively), indicating that each VR is similar in its nerve fiber composition. The relative charge on each root therefore provides a reasonable estimate of the relative number of nerves in that VR that supplies the muscle of interest.

Figure 2.1.

Determination of the relative contributions of L_7 and S_1 VRs to the innervation of MG, LG, and soleus muscles. Methods that use evoked compound action potentials (CAP) on the VRs (B) and tetanic force recorded from MG muscle (C) are illustrated diagrammatically in A. CAPs (in mV) were recorded on each VR separately and then together in response to supramaximal stimulation of the MG nerve and normalized by VR impedance (in kΩ) to derive current (nA) and charge (pC, by integration) (B). Tetanic muscle force recorded in response to stimulation of the L_7 and S_1 VRs provided a complementary estimate of the proportion of motor axons exiting each VR (C). Charge contributions on each VR add to give the total number of motor axons exiting in the root. Similarly, tetanic forces add to equal tetanic force in response to stimulation of the muscle nerve as well as simultaneous stimulation of L_7 and S_1 VRs. In this example, S_1 VR provides an average of 86.5% of the innervation of MG muscle on the basis of force and charge measurements. (Further details are in the text.)


A
Record CAP
Record Impedance
Stimulate
L7
S1
CP
Sc
Stimulate
Record Force
MG
Tib
B
320
4.8
Current (nA)
Voltage (mV)
0
L7 & S1
S1
L7
0.5 ms
C
32
Force (N)
0
L7 & S1
S1
L7
1 sec

The relative contribution of each VR to the innervation of the hindlimb varies considerably from animal to animal, whether determined by charge or force measurements, as shown for eight MG muscles in Table 2.1. Estimates based on charge and force measurements agree reasonably well. The average difference (± S.E.) between the two methods was 10 ± 1.8% (n=8). The 2 estimates were averaged (C+F/2) for each cat and used as the final estimate of the relative number of motor nerves cut in the corresponding root on the contralateral side and how many remained in the uncut VR. In five cats in which charge measurements were not made, force measurements alone were used to estimate the relative unit number.

2.2.3 Quantification of sprouting in PD muscles

The sprouting capacity of motoneurons in the uncut VR and the extent of regeneration of nerves in the cut VR were determined by measuring the total muscle and unit force elicited by stimulation of each VR and teased VR filaments, respectively. Either the MG, LG, or soleus muscle was attached to a force transducer to record whole muscle and single MU force and the MG or LGS nerve was mounted on an array of five silver electrodes to record axon potentials. The electrode array was used in a triphasic configuration to record extracellular axon potentials differentially between the central electrode and the nearest two electrodes (for details, see Gordon et al. 1986b). The outermost electrodes were grounded together to significantly reduce the pickup of electromyogram (EMG) signals from the active muscle (cf. Stein et al. 1977). EMG was recorded from the muscle of interest via bipolar electrodes on a silastic sheet sewn

TABLE 1. Calculated contributions of nerves which exit via S1 ventral roots to the innervation of MG muscles.

	S1 Relative Contribution (%)			
Cat #	Charge	Force	[C-F]	C+F/2
1	2	6	4	4
2	7	20	13	13.5
3	12	14	2	13
4	32	44	12	38
5	35	44	9	39.5
6	46	60	14	53
7	23	14	9	18.5
8	95	78	17	86.5
X ± S.E.	31.5 ± 10.5	35 ± 9.1	10 ± 1.8	33.3 ± 9.6

onto the muscle fascia.

Single MUs were isolated by finely splitting the appropriate VR until a single functional axon to the muscle of interest was identified. The criteria for identifying a single MU were all-or-none single axon, EMG potentials, and unit force at threshold voltage. Ten to 40 MU axon potentials, EMG, and force responses were averaged using a PDP 11/21 microcomputer and stored on disk for further analysis. Signals were monitored throughout the recording on a storage oscilloscope (Tektronix 5441) and a Gould chart recorder. Twitch and maximum tetanic force were elicited by single pulses and trains of 20 pulses at 100 Hz, respectively. MUs were classified physiologically as slow (S), fast fatigue-resistant (FR), and fast fatigable (FF) by their twitch contraction time and fatigability during a 2-min fatigue test (Fleshman et al. 1981; Gordon et al. 1988).

The peak-to-peak amplitude of the triphasic axon potentials recorded on the muscle nerve and conduction velocity were used as electrophysiological measures of axon size. The voltage of the axon potential amplitude was normalized by the mean (± S.E.) nerve-electrode contact impedance (6.1 ± 2.8 KΩ) measured from all experiments so that the potentials could be compared from animal to animal (see Gordon and Stein 1982b). The latency (ms) of the positive peak of the axon potential was used to obtain conduction velocity, with the length between stimulating and recording electrode as the numerator (mm) and the latency as the denominator (ms). MU size was determined from unit force- which was not normalized to whole muscle force or weight, as it has been shown previously that tetanic force is comparable from animal to animal provided that the weight and age of the animals are similar (Gordon et al. 1986b).

2.2.4 Histochemistry and muscle fiber area measurements

Experimental and contralateral muscles were removed immediately after the acute experiment. The muscles were cut into blocks and frozen in isopentane solidified in liquid nitrogen. A small well was created in the frozen isopentane before submersion of the cut muscle blocks to prevent freezing artifact. Muscles were stored at -70°C for subsequent cryostat sectioning, staining and histochemical analysis. Serial cross-sections, 10 μm thick, were cut and stained for myofibrillar ATPase after preincubation at pH 10.4, according to the method of Guth and Samaha (1970), and after preincubation at pH 4.5 according to the method of Brooke and Kaiser (1970) as modified by Green et al. (1982) and described in detail in Gordon et al. (1988). Muscle fibers were histologically classified as Type I, IIA and IIB, however the nomemclature slow-oxidative (SO), fast-twitch oxidative-glycolytic (FOG), and fast-twitch glycolytic (FG) devised by Peter et al. (1973) is used throughout the thesis.

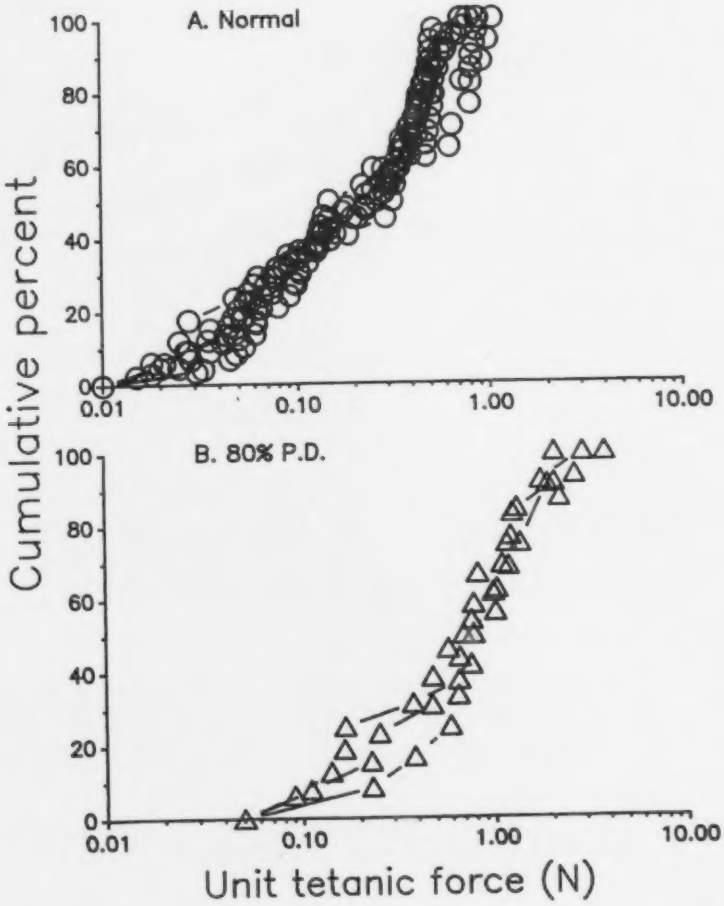
Stained sections were examined with light microscopy and photographs were taken at high magnification (X10) for measurement of muscle fiber CSA. Samples of between 120 and 600 muscle fibers of each type were selected from at least six different areas of the experimental and contralateral control muscles. CSA was calculated from measurements of the outer perimeter of the photographed fibers by use of a digitizing tablet (Summagraphics mm1200 series).

2.2.5 Statistical analysis

Seven to 24% of the total MU population in MG, LG, and soleus muscles was sampled in control and experimental animals for comparison of electrophysiological parameters of MU and nerve size, namely isometric force, axon potential amplitude, and conduction velocity. Comparisons were made by constructing cumulative frequency histograms for each parameter of the MU sample in each animal. Differences between cumulative distributions for different muscles were tested for significance using the nonparametric Kruskal-Wallis test (Sokal and Rohlf 1969), which tests for differences in the rank order of the parameter in each distribution. If the distributions from different animals were not significantly different at the 95% confidence level, MU samples were pooled together. Two examples are provided in Fig. 2.2. The distributions of unit tetanic force in MG muscles from six normal animals are plotted on semi-logarithmic scales in Fig. 2.2A, where the distributions clearly overlap and are not statistically different. Similarly, in Fig. 2.2B, distributions of unit force from three MG muscles that lost 75, 80 and 82% of the innervation by section of the S_1 VR are not significantly different. As a result these samples from partially denervated muscles could be pooled. Data from muscles that lost 8-95% of their innervation were compared and data pooled when cumulative distributions were not significantly different. The pooled data were collected by selecting logarithmic bin intervals to represent the distribution of logarithmic force values (Fig. 2.3) and arithmetic bin intervals to represent the distribution of axon potential amplitudes and conduction velocities (Fig. 2.11). The force on the X-axis is shown in logarithmic intervals of equal bin width and not of the exponential values used

Figure 2.2.

Cumulative distributions of between 12 and 51 single MG MU tetanic forces in 6 individual normal muscles are compared in (A) and distributions in 3 partially denervated muscles in which ~80% (78 ± 1.7%) of the innervation was removed are compared in (B). Rank order analysis, using the Kruskal-Wallis test, showed that the distributions plotted within (A) or (B) are not significantly different from each other ($P<0.01$), however the distributions within A are significantly different from those within B ($P<0.01$).


A. Normal
B. 80% P.D.
Cumulative percent
Unit tetanic force (N)
100
80
60
40
20
0
0.01
0.10
1.00
10.00

to calculate the bin intervals in order that the actual force values can be more readily visualized (Figs. 2.3 and 2.4). Significant differences between the pooled cumulative distributions were tested by applying the Kolmogorov-Smirnov test (Fisz 1963). Differences between mean values were analyzed using the Student's *t* test. Mean values ± standard error values (mean ± S.E.) are shown together throughout except where geometric means were calculated.

Regression lines were fitted according to a least-mean-squares method, and correlation coefficients (RO) were calculated according to standard equations for a linear regression (Hartley 1961).

2.3 RESULTS

2.3.1 Partial denervation

By sectioning one of two contributing spinal roots, we reduced the number of MUs in MG, LG, and soleus muscles. The motor innervation to 8 MG muscles was reduced by an average (± S.E.) of 33.3 ± 9.6% as determined from the mean relative charge and tetanic force contributions of the VR to the muscles in the contralateral unoperated hindlimb (Table 2.1; see METHODS). The total charge contribution of S_1 and L_7 VRs to innervation of MG muscles is 190 ± 41 pC (mean ± S.E.) of which an average of 31.5% is contributed by S_1 VR. MG muscles develop an average tetanic force of 59 ± 8.5N (n=8), of which stimulation of S_1 VR elicits 35% of the total force on average. The relative contribution of each VR to the innervation of the triceps surae muscles varies considerably between animals. For example, section of the S_1 spinal root removed between 4 and 86.5% of the MG muscle innervation (Table 2.1). Similarly, between 8 and 95% of the innervation of the LG and soleus muscles was removed by section of either S_1 or L_7 in six and three cats, respectively.

2.3.2 Enlargement of remaining MUs in partially denervated muscles

2.3.2.1 Units in muscles in normal adult cats

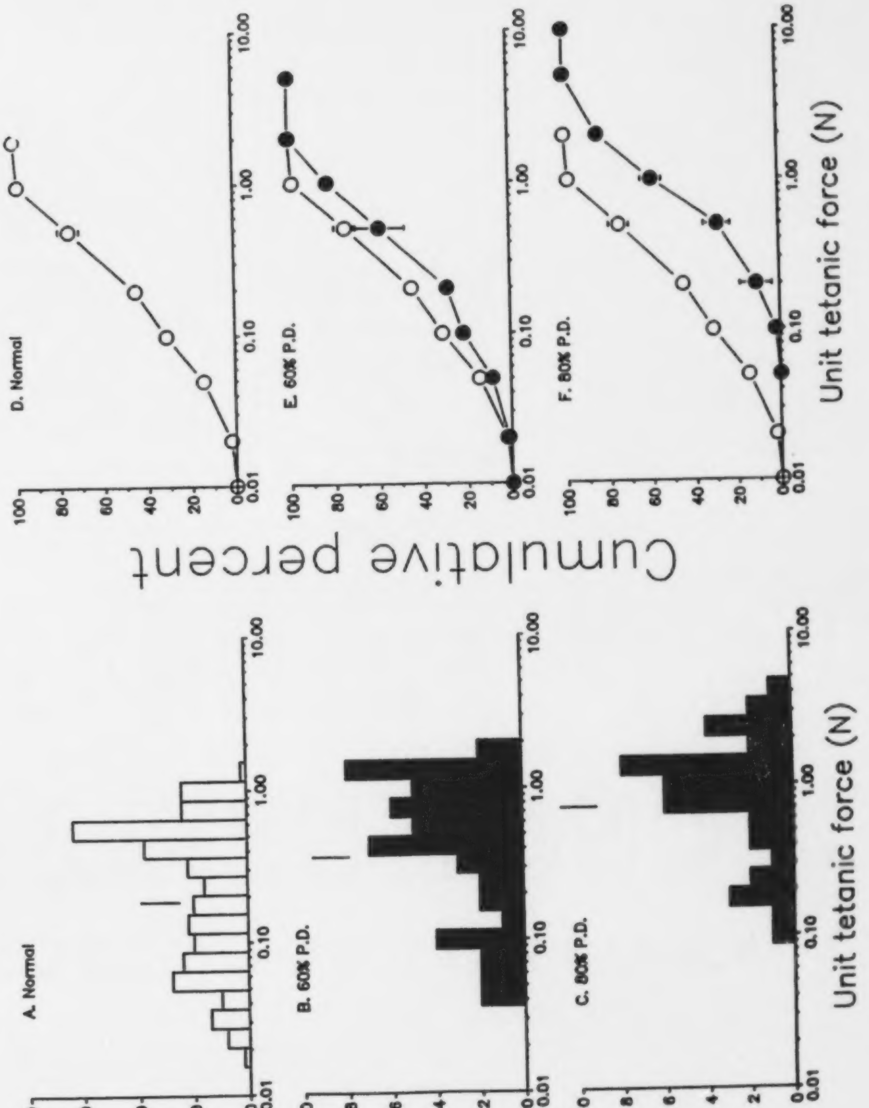
MU tetanic force normally varies over a 100-fold range in MG and LG muscles and over a 5-fold range in soleus muscle with mean (± S.E.) forces of 307 ± 18, 360 ± 73 and 85 ± 10.1 mN, respectively. The arithmetic distribution for MG unit tetanic

TABLE 2 Reinnervation of partially denervated muscle by cut ventral root nerve fibers after ventral root section in the adult and neonatal animal.

		Innervation		
Duration (months)	% PD	%	N	Muscle
ADULT				
4.6 ± 0.72	10-85	0	8	MG & LG
3.5	85	40	1	MG
6	93	45	1	LG
3	98	79	1	MG
NEONATE				
13.5	30-69	0	2	MG & LG
18	69	50	1	LG
14.5	80	19	1	MG
10.5	88	23	1	LG
7	94	44	1	LG

Figure 2.3.

Frequency histograms (A-C) and cumulative probability distributions (D-F) of MG unit tetanic forces in normal muscles (open histograms and open circles) and partially denervated muscles (PD) in which ~60% (58 ± 5%, n=2), and ~80% (79 ± 2.0%, n=3) of innervation was removed by cutting either S_1 or L_7 spinal roots (filled histograms and circles). Normal bimodal distribution of unit force is shifted to larger force values after PD without changing the range or shape of the distributions. Geometric means (0.19, 0.34 and 0.72 N in A,B, and C, respectively), shown as vertical bars, are significantly different ($P<0.01$). Values shown in the cumulative histograms in D-F are means ± S.E. where the S.E.'s are visible only when larger than the symbol.


A. Normal
B. 60% P.D.
C. 80% P.D.
Unit number
40
30
20
10
0
10
8
6
4
2
0
0.01
0.10
1.00
10.00
Unit tetanic force (N)
D. Normal
E. 60% P.D.
F. 80% P.D.
Cumulative percent
100
80
60
40
20
0
0.01
0.10
1.00
10.00
Unit tetanic force (N)

forces is skewed to the right because there are more small MUs than large (Henneman and Mendell 1981), with the result that logarithmic values of forceare more normally distributed (Fig. 2.3A). In cat MG muscles, which contain a high proportion of slow MUs (Burke 1981), the distribution is bimodal (Figs. 2.2A and 2.3D). In the cat soleus muscle, which is composed exclusively of slow MUs, the logarithmic distribution of MU tetanic force is unimodal, corresponding well with the first mode in the MG unit force distribution (cf. Fig. 2.4A and C and Fig. 2.3A and D).

2.3.2.2 Units in muscles partially denervated in adult cats

The MG unit force distribution remains bimodal after PD but shifts to the right to larger force producing MUs. Reduction of 60% (X ± S.E.: 58 ± 5%, n=2) and 80% (79 ± 2.0%, n=3) of the MU population shifted the distribution to larger values but did not significantly change the range or shape of the frequency histograms (cf. Fig. 2.3A-C). This is more clearly seen when the distributions are plotted as cumulative frequency histograms (Fig. 2.3D-F) where a parallel shift in the distribution to the right after PD (filled symbols) indicates that force in all units increases by the same factor. Thus, for example, 50% of units generate tetanic force <0.2N in normal muscles (Fig. 2.3D), <0.4N in muscles where 60% of innervation was removed (Fig. 2.3E), and <0.8N after 80% PD (Fig. 2.3F).

Similarly, all MUs in soleus muscles contributed to sprouting because the unit force distributions were not altered in shape or range (Fig. 2.4A and B) and cumulative frequency histograms were shifted in parallel to larger values (Fig. 2.4C and D). The

Figure 2.4.

Comparison of frequency histograms (A and B) and cumulative probability distributions (C and D) of unit tetanic force in a normal soleus muscle (open histogram and open circles) and partially denervated muscles in which 70% (shaded histogram; A and filled circles; C) and 90% (shaded histogram; B and filled triangles; D) of the innervation was removed by either L_7 (A and C) or S_1 (B and D) spinal root section. Geometric means for the normal (vertical line) and enlarged (vertical with asterisk) soleus MUs following 70% and 90% PD were 0.081, 0.398, and 0.389 N, respectively. The soleus unit distributions after 70 and 90% PD were not significantly different ($P<0.01$).

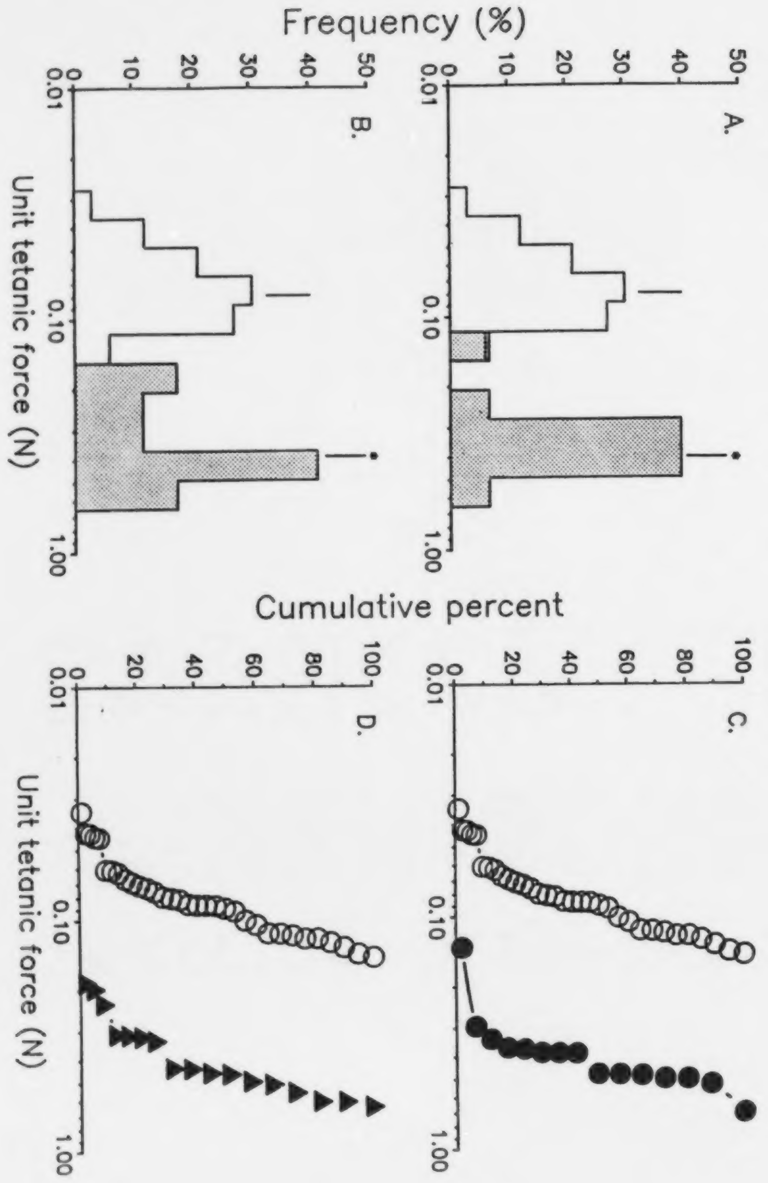
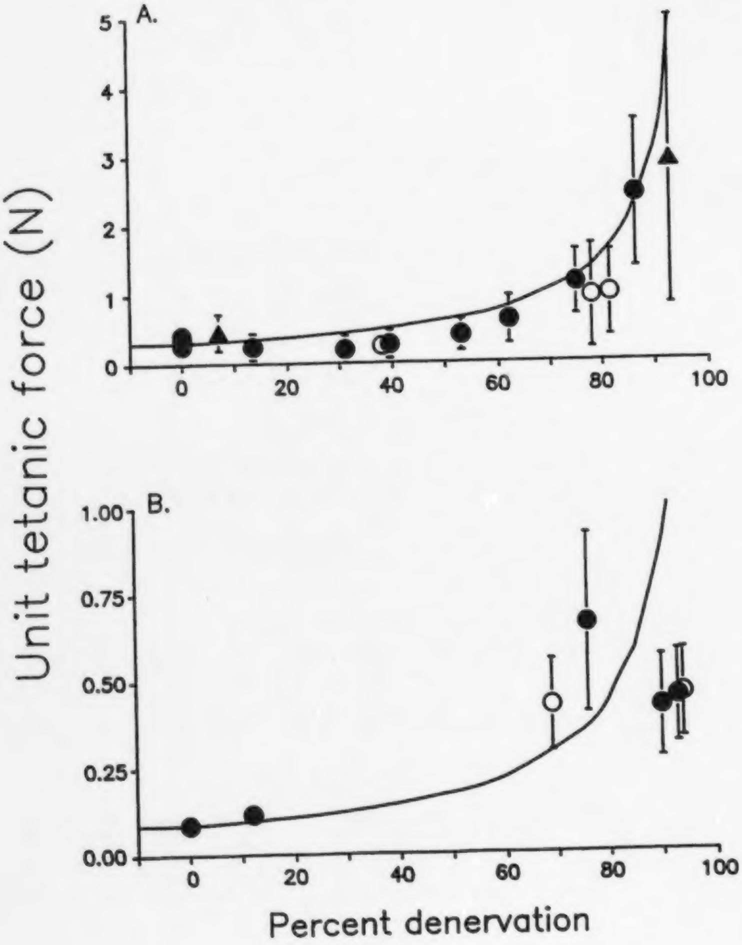

Frequency (%)
A.
B.
0
10
20
30
40
50
0.01
0.10
1.00
Unit tetanic force (N)
Cumulative percent
C.
D.
0
20
40
60
80
100
0.01
0.10
1.00
Unit tetanic force (N)

Figure 2.5.

Mean unit tetanic force (± S.E.) in MG (filled circles), LG (filled triangles) (A) and soleus (B) muscles are plotted against extent of PD (%) and compared with a predicted curve for the condition where unit force (UF_c; curved line) increases in direct proportion to the percent (% PD) calculated as a function of $UF_c/(1-\%PD)$ where UF_c is the mean unit force in normal muscles (see RESULTS for details). Data from muscles that were partially denervated at 3-4 weeks of age are shown as open symbols.


A.
5
4
3
2
1
0
0
20
40
60
80
100
B.
1.00
0.75
0.50
0.25
0.00
0
20
40
60
80
100
Unit tetanic force (N)
Percent denervation

shift was proportional to the extent of PD for muscles in which 70% of innervation was removed (Fig. 2.4A,C), but there was no further shift for muscles with more extensive PDs (cf. Fig. 2.4B and D). The distributions of unit force in all soleus muscles that lost more than 70% of their innervation were not statistically different ($p<0.05$). Unit force of all these muscles increased by a factor of 4.

2.3.2.3 Units in muscles partially denervated in kittens

When muscles were partially denervated in kittens at 3-4 weeks of age, the time when the muscle fibers share innervation with two to three motoneurons (Bagust et al. 1973), the final MU size measured 6-18 months later was directly comparable with MUs sampled in muscles that were partially denervated to the same extent during adulthood. Statistical comparisons of the frequency distributions of unit force in MG and soleus muscles that lost the same proportion of innervation by neonatal and adult spinal nerve section showed that the distributions were not statistically different ($p<0.01$). The mean values are compared in Fig. 2.5, where open symbols are the values for MUs sampled in muscles partially denervated in kittens. The final MU size attained in partially denervated MG (compare open and closed symbols for 40 and 80% PD in Fig. 2.5A) and soleus (Fig. 2.5B) muscles is the same irrespective of the initial MU size or the maturity of the neuromuscular system.

2.3.2.4 Proportional but limited MU enlargement

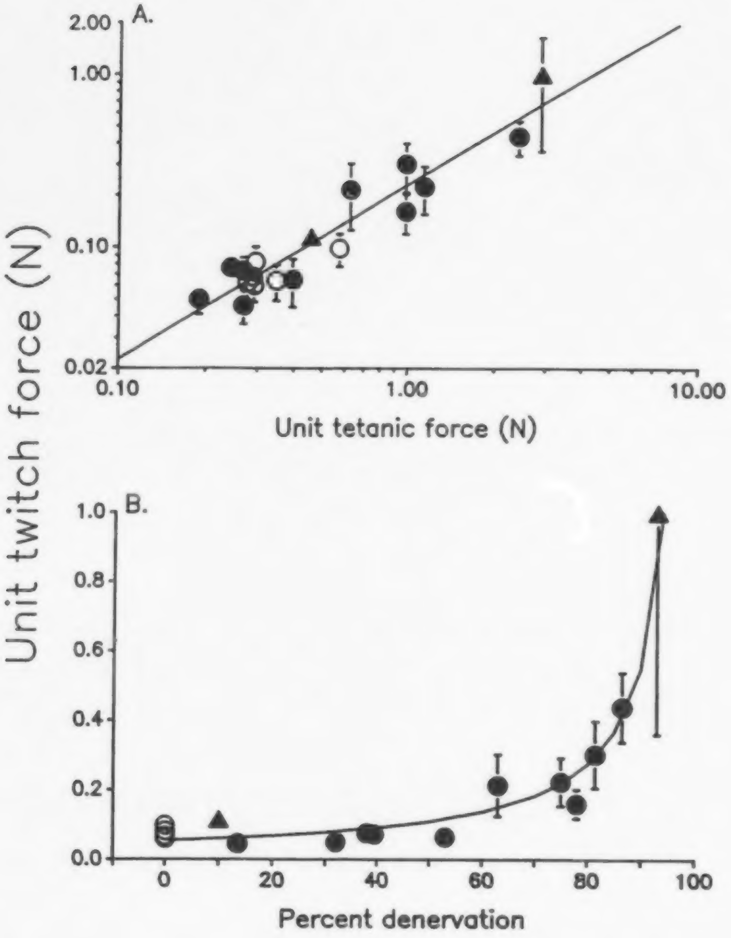
If the force of remaining MUs increases in proportion to the reduction in their number in partially denervated muscles, and the sprouts reinnervate all available denervated muscle fibers, unit force (UF_e) will increase as a function of $UF_c/(1\text{-}PD)$, where UF_c is the mean unit force of MUs in normal muscles and PD is the fraction of MUs lost after ventral root section. As shown in Fig. 2.5 the prediction of UF_e was met for muscles with progressively higher PD, but unit force fell short for extensively denervated muscles ($\geq$90%). Regenerated nerves made functional contacts in gastrocnemius but not soleus muscles. The relatively small number of MUs sampled, as well as reinnervation and displacement of sprouted terminals by regenerating nerves from the cut VR (Table 2.2; see below), are both likely to account for the large S.E. in the mean force of MUs in extensively denervated muscles. In extensively denervated soleus muscles, the increase in MU size of 4-5 times could not compensate fully for the PD (see also Luff et al. 1988) with the result that the muscles did not fully recover and their force was significantly less than normal (11 ± 4.8N compared with 26.7 ± 4.1N).

2.3.2.5 Increase in unit twitch force

Although maximum tetanic force is generally a more reliable measure of unit force than twitch force (Burke 1981), many previous studies of partially denervated muscles have measured twitch rather than tetanic force (e.g. Brown and Ironton 1978; Fisher et al. 1989; Thompson and Jansen 1977). Within MU populations of normal and partially denervated muscles, unit twitch force increases as a direct function of **Figure**

Figure 2.6.

Mean (± S.E.) unit twitch force (potentiated after 6 tetanic contractions) of normal (open circles) and partially denervated MG (closed circles) and LG (closed triangles) muscles plotted as a function of tetanic force (A) and percent PD (B). Slope (± S.E.) of the regression in A is 1.02 ± 0.08, RO=0.9. Curve in B is calculated as described for Fig. 2.5.


A.
2.00
1.00
0.10
0.02
0.10
1.00
10.00
Unit tetanic force (N)
Unit twitch force (N)
B.
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
Percent denervation

tetanic force: the slope of regression lines plotted on double logarithmic curves is unity. This is clear when the mean values of twitch and tetanic forces of normal (open symbols) and partially denervated MG and LG muscles (filled symbols) are plotted on double logarithmic axes in Fig. 2.6A. It is, therefore, not surprising that twitch unit force increases as a function of percent denervation (Fig. 2.6B) in the same way as observed for unit tetanic force (Fig. 2.5A).

2.3.2.6 Response of MU types

Individual unit tetanic force values are plotted separately for each physiological MU type (Fig. 2.7), fitted with the same predicted curve as in Figs. 2.5 and 2.6B, and compared with the mean force of S, FR, and FF MUs in normal muscles. In these plots, which are similar to those used by Luff et al. (1988), it is readily seen that the force of any individual MU, relative to the mean unit force in normal muscles (dotted line), is widely scattered. A MU may be up to 10.5 times larger than the mean as shown for one sprouted FR unit (indicated by a * in Fig. 2.7C) in a muscle that lost 86.5% of its innervation. Thus, when force of any one MU is compared with the mean unit force, without consideration of the location of the MU within the population distribution, the relative increase in MU size may be incorrectly evaluated. The consequences of this evaluation of MU enlargement are considered in detail in the DISCUSSION.

2.3.2.7 Contribution of muscle fiber area to MU size

Unit force is a reliable index of IR after PD if the other contributing variables to force, namely muscle fiber CSA and specific force, are not altered. Specific force is normally a minor contributing factor that does not change after reinnervation (Tötösy de Zepetnek et al. 1992), whereas CSA, on the other hand, makes a significant contribution in determining the unit force range in normal muscles (see Bodine et al. 1987; Tötösy de Zepetnek et al. 1992). CSA increases in the order SO=FOG<FG in normal muscles, although there is considerable overlap of the fiber type distributions even for the FG fibers, as shown for two MG muscles in Fig. 2.8 (see also Gordon et al. 1988). Similar distributions were observed in muscles after PD removed <70% (Moderate PD) of the innervation (Fig. 2.8A-C). However, the distribution of SO and FOG fibers tended to move to larger values and FG fibers to move to smaller values with corresponding changes in their mean CSA values (shown as vertical lines in Fig. 2.8). These changes become evident when >75% (high PD) of the innervation is removed and differences between SO and FOG fibers with FG fibers are lost (Table 2.3; Fig. 2.8D-F).

As the extent of muscle PD increases, the mean CSAs of the different fiber types become more similar in size with a concurrent increase in the standard deviation, resulting in an increased variability in the size of the three fiber types (Table 2.3). One muscle which was reinnervated after cutting both S_1 and L_7 VRs (100% PD) is included in the table to show that all three types are similar in size after reinnervation (see also Foehring et al. 1986), an effect very similar to that seen after cross- reinnervation of this muscle (Gordon et al. 1988). This trend to equality is shown in Fig. 2.9. The mean CSA of SO fibers increases and of FG fibers decreases in size, with progressively more extensive PDs (Fig. 2.9A-C) resulting in all fiber types becoming similar in size **Figure**

Figure 2.7.

Tetanic force of slow units in MG muscle (A), slow units in soleus muscle (B), FR units (C) and FF units (D) in MG muscle plotted as a function of the extent PD (%) and compared with normal mean unit force values (dotted lines) and the expected increase in mean unit force shown as solid lines (see Fig. 2.5 and RESULTS for details). Normal mean unit force values (± S.E.) are 65 ± 5 (A), 87 ± 25 (B), 222 ± 25 (C), and 525 ± 15 (D) mN. Note that any one MU may be up to 10.5 times larger than the mean (indicated by *; C) but that this MU is within 2-3 SDs from the mean in the frequency distribution of unit force (cf. Fig. 2.3).

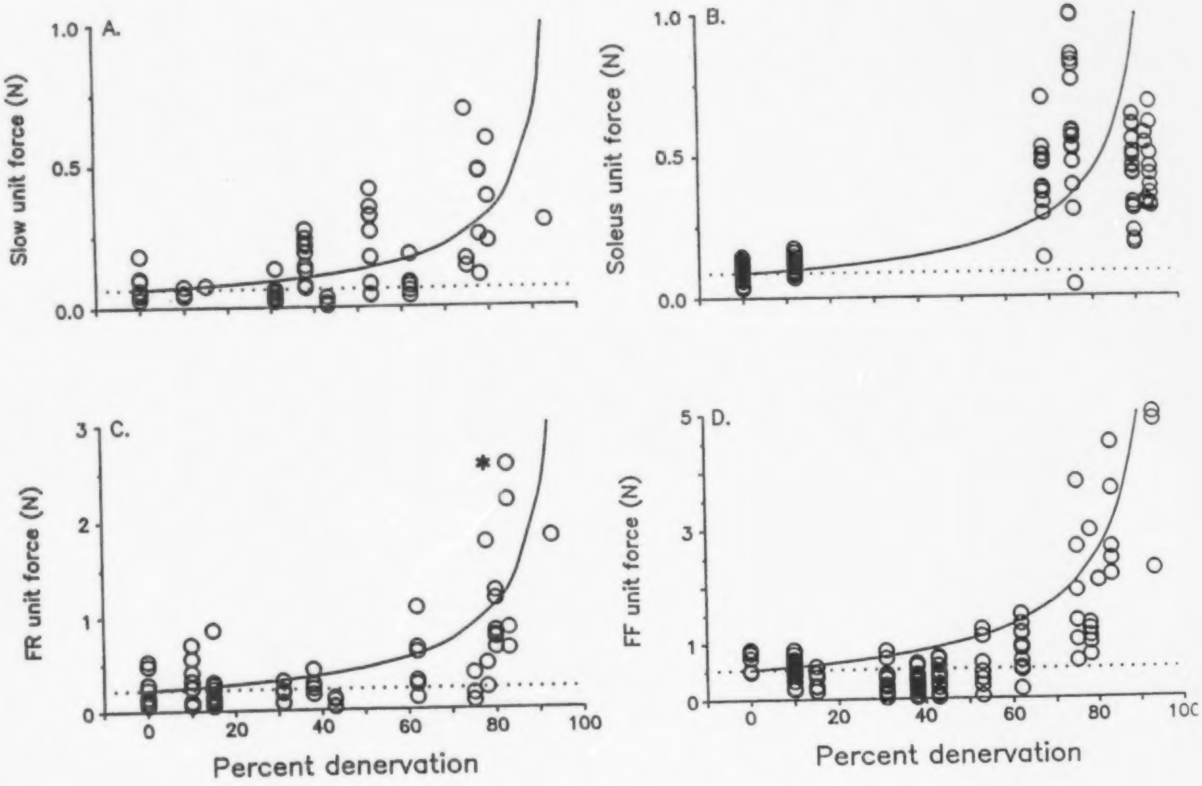
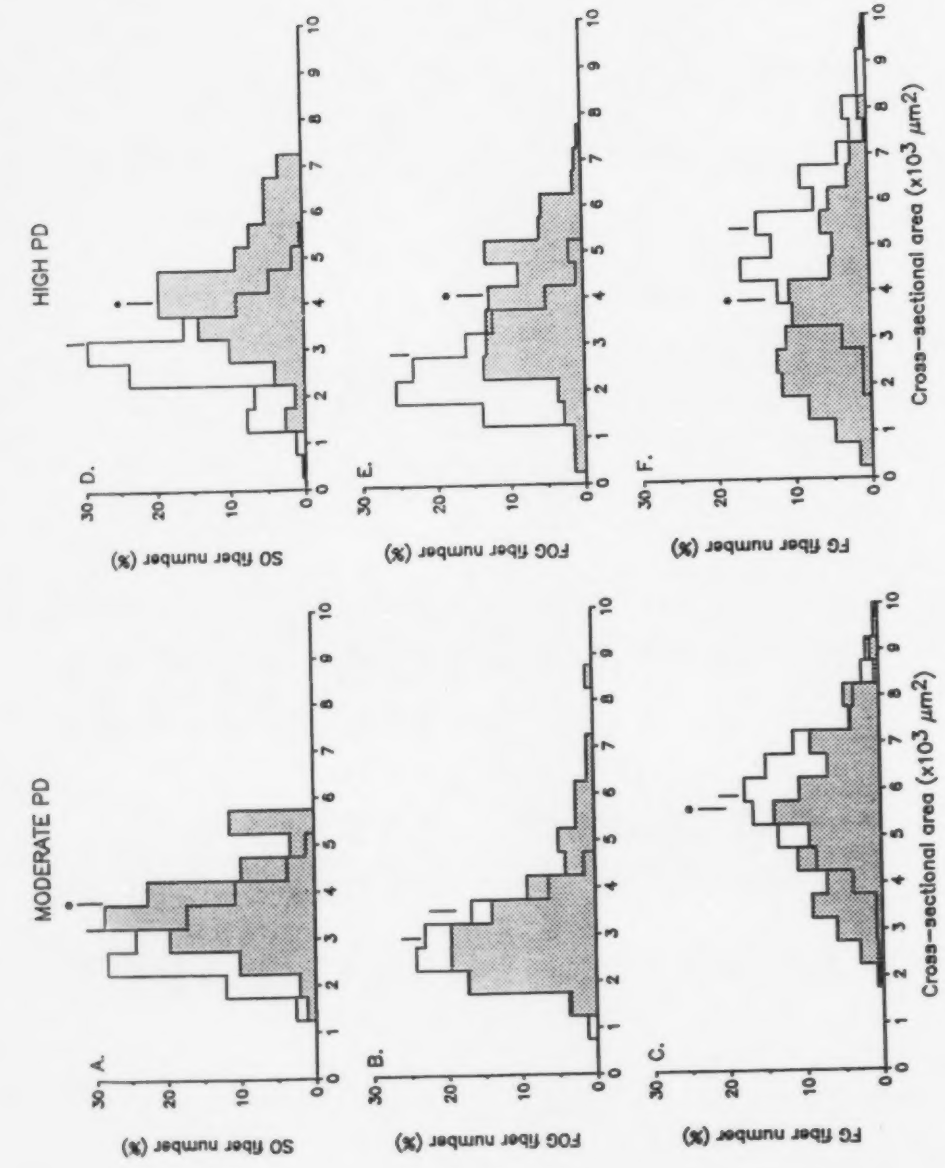

A.
Slow unit force (N)
1.0
0.5
0.0
B.
Soleus unit force (N)
1.0
0.5
0.0
C.
FR unit force (N)
3
2
1
0
*
0
20
40
60
80
100
Percent denervation
D.
FF unit force (N)
5
3
1
0
0
20
40
60
80
100
Percent denervation

Figure 2.8.

Frequency histograms comparing the CSA of SO, FOG, and FG muscle fibers in normal MG muscles (open histograms), with fibers in the contralateral experimental muscle (shaded histograms) which were partially denervated by 50% (moderate PD; A-C), 4 months previously, and by 75% (high PD; D-F), 3.5 months previously, after unilateral section of S_1 spinal root. Mean CSA values (± S.E. μm^2) of each fiber type in normal (vertical line) and 50% PD (vertical line with asterisks) muscles are similar, being 3214 ± 49 and 3763 ± 54 for SO (A), 2912 ± 44 and 3486 ± 111 for FOG (B), and 5845 ± 53 and 5685 ± 83 for FG (C). Means (± S.E. μm^2) for normal and 75% PD muscles are 3202 ± 49 and 4046 ± 95 (D), 2798 ± 53 and 4035 ± 90 (E), 5371 ± 53 and 3757 ± 97 for SO, FOG and FG fibers, respectively, and are all significantly different ($P<0.01$).


MODERATE PD
HIGH PD
A.
B.
C.
D.
E.
F.
SO fiber number (%)
FOG fiber number (%)
FG fiber number (%)
0
10
20
30
0 1 2 3 4 5 6 7 8 9 10
Cross-sectional area (x10³ μm²)
*

with a mean ($\pm$ S.E.) of 3942 $\pm$ 40 μm^2 (n=12) in extensively denervated muscles (>80%). The final outcome is that CSA makes a significant contribution to the increase in S unit force, but not the fast units (cf. Fig. 2.9D-F). The increased contribution of CSA to determining S unit force indicates that force measurements slightly overestimate the sprouting capacity of S units. The small reduction in FG muscle fiber size results in a slight underestimate of IR from force measurements for the FF units. The force measurements for the FR units, on the other hand, represent the change in their IR after PD reasonably well. These trends are seen in Fig. 2.7 where individual S unit forces slightly exceed predicted values, FR values are well fitted by the predicted curve, and FF unit forces tend to be underestimated. However, in contrast to gastrocnemius muscles, S units in soleus muscles do not reinnervate any fibers other than SO fibers therefore the SO fiber CSA was not different from normal, even in the most extensively denervated muscles (Fig. 2.10).

2.3.3 Size relationships among MU force, contractile speed and motor nerve size in partially denervated muscles

2.3.3.1 Motor nerve size

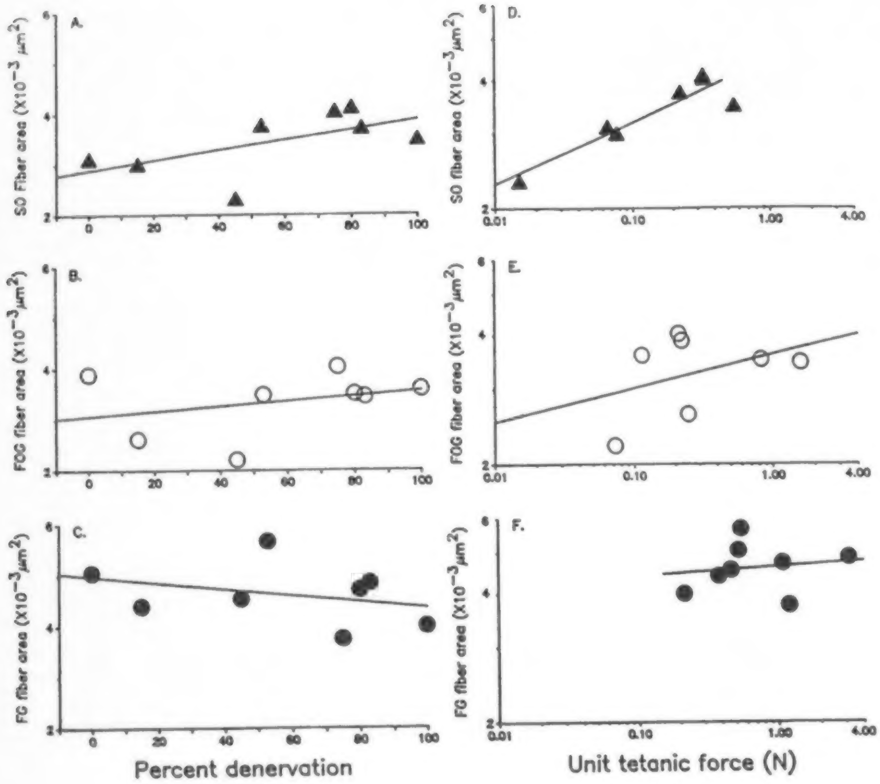
Peak to peak amplitude of evoked unitary nerve potentials recorded on the MG muscle nerve varies over a fourfold range compared with a twofold range for conduction velocity (Fig. 2.11A and B), consistent with the findings that potential amplitude varies as the fourth power and conduction velocity varies as the square of the fiber diameter (Gordon and Stein 1982b; Stein et al. 1977). The 4-6 times increase in the number of

TABLE 3. Comparison of muscle fiber cross-sectional area in partially denervated and the contralateral MG muscles.

	NORMAL: FIBER CROSS-SECTIONAL AREA			PD:FIBER CROSS-SECTIONAL AREA		
	SO	FOG	FG	SO	FOG	FG
% PD	X ± S.D.	X ± S.D.	X ± S.D.	X ± S.D.	X ± S.D.	X ± S.D.
0	2903 ± 628	2648 ± 687	5041 ± 1225	-	-	-
13	3054 ± 588	2928 ± 844	4901 ± 1190	3010 ± 622	2613 ± 744	4404 ± 1210
45	2354 ± 438	2172 ± 468	4684 ± 1216	2318 ± 738	2210 ± 534	4550 ± 1273
53	3214 ± 681	2912 ± 683	5845 ± 1033	3763 ± 729	3486 ± 1214	5685 ± 1681
75	3202 ± 803	2798 ± 789	5371 ± 1430	4046 ± 1211	4035 ± 1332	3757 ± 1705
80	-	-	-	4118 ± 970	3506 ± 887	4723 ± 1587
86.5	3617 ± 692	3323 ± 937	6245 ± 1739	3716 ± 1018	3453 ± 1229	4862 ± 1863
100	-	-	-	3486 ± 1076	3594 ± 1055	4003 ± 1229

Figure 2.9.

Mean values of muscle fiber CSA for each fiber type are plotted as a function of the extent PD (A-C) and as a function of mean unit tetanic force (D-F) plotted logarithmically. SO fiber size (A) tends to increase and FG fiber size (C) to decrease with progressive denervations, although slopes of regression lines were not significantly different from 0 (RO: 0.57 in A, 0.27 in B, and 0.36 in C). Force and CSA covary only in SO fibers (D) where the slope (± S.E.) of the regression line is 0.15 ± 0.03 with a RO=0.91, which is significantly different from 0 ($p<0.01$) in contrast to the slope of regression lines fitted in E and F.


A.
SO Fiber area (X10^{-3} μm^2)
B.
FOG fiber area (X10^{-3}μm^2)
C.
FG fiber area (X10^{-3}μm^2)
D.
SO fiber area (X10^{-3}μm^2)
E.
FOG fiber area (X10^{-3}μm^2)
F.
FG fiber area (X10^{-3}μm^2)
2
4
6
0
20
40
60
80
100
0.01
0.10
1.00
4.00
Percent denervation
Unit tetanic force (N)

muscle fibers supplied by the uncut nerve did not have a significant effect on the size of the motor nerve because the range and mean values of axon potential amplitude and conduction velocity for these nerves were not significantly different from normal.

2.3.3.2 Size relationships

Unit force normally covaries with axon potential amplitude and contractile speed, as shown for MUs sampled from individual MG muscles (Fig. 2.12A and D). The slopes (± S.E.) of the regression lines, 0.16 ± 0.04 and -0.12 ± 0.04, are significantly different from zero ($p<0.01$) and very similar to published values (Gordon and Stein 1982a; Gordon et al. 1986b). After PD these relationships were not altered, as shown by the similar slopes of the regression lines fitted to the data for muscles in which the MU numbers were reduced by 60% (Fig. 2.12B and E) and 80% (Fig. 2.12C and F). The increase in the MU size, without retrograde effects on the motor nerves, is seen as a shift of the curves to the right along the force axis in the partially denervated muscles, with no shift in the Y-axis. The average values (± S.E.) of the slopes of the computed regressions of axon potential amplitude and twitch contraction time on unit force are 0.15 ± 0.05 and -0.20 ± 0.02, respectively, for nine PD muscles compared with 0.14 ± 0.01 and -0.15 ± 0.012 for six normal muscles. Similar results were obtained for LG and soleus muscles and in MUs measured in muscles that underwent PD soon after birth.

Figure 2.10.

Frequency histogram comparing the CSA of muscle fibers in 3 normal soleus muscles (open histogram) and muscle fibers in 4 contralateral experimental muscles with a mean (± S.E.) PD of 86.5 ± 5.9% (shaded histogram). Mean CSA values (± S.E.) are 4305 ± 54 and 3939 ± 43 for normal (vertical line) and partially denervated (vertical line with asterisks) soleus muscles, respectively.

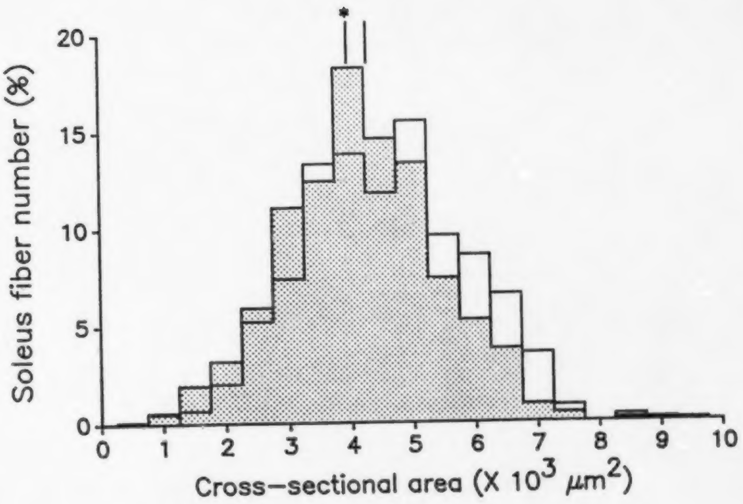
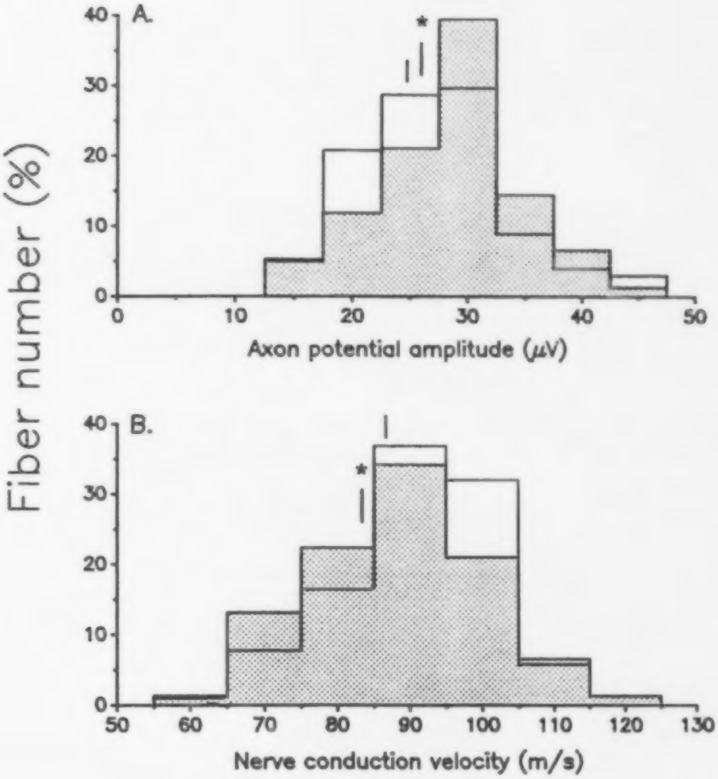

*
Soleus fiber number (%)
20
15
10
5
0
0
1
2
3
4
5
6
7
8
9
10
Cross—sectional area (X 10^3 μm^2)

Figure 2.11.

Frequency histograms of peak-to-peak amplitude of axon potentials (A) and conduction velocities (B) of nerves that supply normal (open histogram: $n=3$) and partially denervated (shaded histogram: 70 ± 6.5%, $n=4$) MG muscles. Distribution and mean (± S.E.) values are not significantly different ($p<0.01$). Mean values for the partially denervated muscles are indicated by the vertical line with asterisks.


Fiber number (%)
A.
40
30
20
10
0
0
10
20
30
40
50
Axon potential amplitude (μV)
*
B.
40
30
20
10
0
50
60
70
80
90
100
110
120
130
Nerve conduction velocity (m/s)
*

2.3.4 Interaction of sprouting and regenerating nerve terminals

2.3.4.1 Reinnervation of partially denervated muscles by cut VRs

Three out of 11 gastrocnemius muscles in which a spinal root was cut in the adult cat had substantial reinnervation of the partially denervated muscles: 40, 45, and 79% of the total muscle force was elicited by cut motor axons that had regenerated and reinnervated muscle fibers (Table 2.2). Four out of six cut VRs successfully reinnervated muscle fibers when the VR was cut in the first month of life. Between 19 and 50% of the total force was contributed by MUs supplied by the cut VR, which made functional connections. Forces elicited by cut and uncut VRs were additive, with no evidence of occlusion, showing that regenerated nerves had either successfully displaced sprouted terminals from the end-plates supplied by the intact nerve or had selectively reinnervated denervated muscle fibers. Soleus muscles were rarely reinnervated by the cut VR after root section in either neonatal or adult cats (see DISCUSSION).

2.3.4.2 Duration and extent of PD

It is unlikely that the time delay between spinal root section and measurement of unit force could account for the success with which regenerating nerves reinnervated and displaced sprouted terminals because the mean time (± S.E.) was 4.2 ± 0.93 months (range 3.5-6, n=3) for the adult animals in which muscles became reinnervated and 4.6 ± 0.72 months (range 3-9, n=8) for those that did not. The delay averaged 12.5 ± 2.4 months (range 7-18, n=6) for cats in which roots were cut in the neonate, and, again, the degree of displacement of sprouted terminals did not correlate with time. On the other hand, where more than 80% of the innervation was removed by cutting the S_1 root,

Figure 2.12.

Axon potential amplitude and twitch contraction time plotted as a function of tetanic force in MUs from a normal (A and D), 60% PD (B and E) and 80% (C and F) MG muscles. Slopes (± S.E.) of regression lines for axon potential amplitude and tetanic force (0.16 ± 0.04, 0.15 ± 0.04, 0.15 ± 0.03; RO=0.63, 0.59, 0.83, respectively) are all significantly different from 0 ($P<0.01$-0.05) and not different from each other. Negative slopes of regression lines for contraction time and tetanic force (0.12 ± 0.04, 0.21 ± 0.3, 0.16 ± 0.05; RO=0.52, 0.77, 0.68, respectively) are also similar for normal and partially denervated muscles.

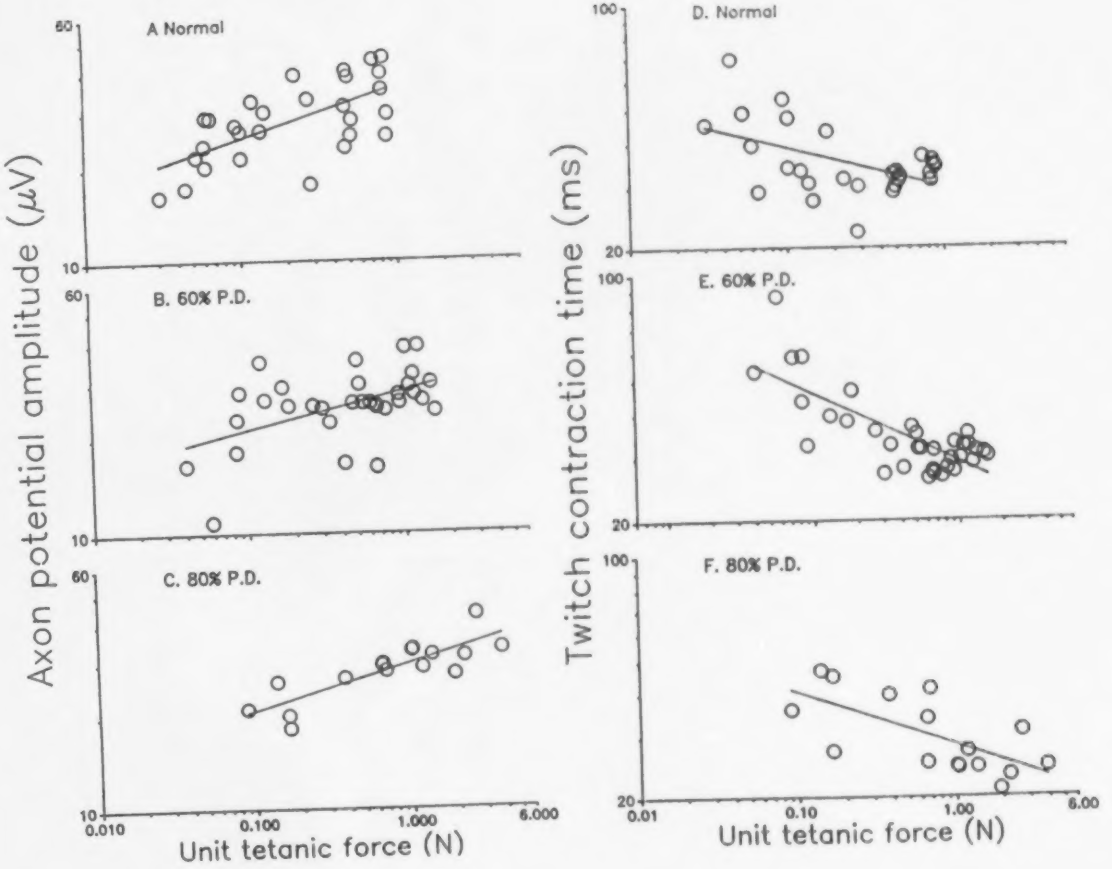
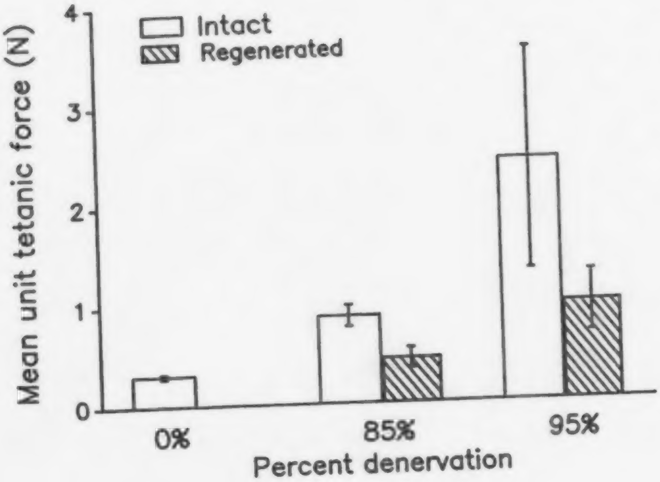

A Normal
B. 60% P.D.
C. 80% P.D.
D. Normal
E. 60% P.D.
F. 80% P.D.
Axon potential amplitude (μV)
Twitch contraction time (ms)
Unit tetanic force (N)
Unit tetanic force (N)
60
10
60
10
60
10
100
20
100
20
100
20
0.010
0.100
1.000
6.000
0.01
0.10
1.00
6.00

Figure 2.13.

Mean (± S.E.) MG and LG unit tetanic forces in normal and partially denervated muscles supplied by nerves in the intact VR (open bars) and cut and regenerated VR (crossed-bars). Regenerating nerves did not make functional nerve-muscle connections for PD $<80\%$ in muscles partially denervated in the adult cat. Where functional contacts were made, MUs were significantly smaller than MUs supplied by the intact VR ($p<0.01$).


Intact
Regenerated
Mean unit tetanic force (N)
4
3
2
1
0
0%
85%
95%
Percent denervation

displacement of sprouted terminals occurred in every animal but one.

2.3.4.3 Size of reinnervated units severed by cut root

When terminals from regenerating nerves successfully displaced sprouted terminals, the MUs were significantly smaller than units supplied by the sprouted terminals from intact motoneurons, however, in extensively partially denervated muscles, the average MU size was larger than normal (Fig. 2.13). Interestingly, soleus muscles were almost never reinnervated by regenerating nerves, possibly because all the regenerating nerves made functional contacts with LG muscle fibers, which they encountered first.

2.4 DISCUSSION

In addition to providing confirmatory evidence that average MU size can increase to 4-6 times its normal size in PD muscles, the results of these studies show clearly, and for the first time, that all MUs contribute to the sprouting, with the relative increase in MU size being the same. These data further show that there is no retrograde influence on motor nerve size of the enlarged MU and that the increase in IR by collateral sprouting occurs in direct proportion to the size of the innervating motor nerve.

2.4.1 MU size

Studies in which the IR has been determined directly in glycogen-depleted MUs (Bodine et al. 1987; Burke et al. 1973; Chamberlain and Lewis 1989; Cope et al. 1986; Tötösy de Zepetnek et al. 1992) indicate that unit force is a reasonable measure of MU size, provided that muscle fiber CSA is not greatly affected by the experimental procedure or developmental changes. However, many studies have relied on measurements of unit twitch force, which is a less reliable parameter of force capacity because of differences in tetanic to twitch ratios and susceptibility of the twitch contraction to facilitation or depression (Burke 1981; Close 1972, reviewed by Jansen and Fladby 1990; Thompson 1985). Nevertheless there may be reasonable agreement between unit twitch and tetanic force measurements (Fig. 2.6A), even when these are carried out for the same MUs under chronic and acute recording conditions (Gordon and Stein 1982ab).

With the exception of studies by van Harreveld (1945) and Luff et al. (1988), all studies agree that MU size can increase to up to 4-6 times the normal adult size (this study; Brown and Ironton 1978; Fisher et al. 1989; Gorio et al. 1983; Thompson and Jansen 1977; Yang et al. 1990). Luff and colleagues (1988) claimed that MUs can increase in size by as much as 19 times in partially denervated flexor digitorum longus (FDL) muscle of the cat and 16 times in soleus muscles. Nevertheless, they concluded that most MUs increase in size between 2 and 7 times that of normal, which is in good agreement with the upper limit of 4-6 times discussed above. The infrequent but very large increase in unit size may simply be accounted for by their method of comparison of the force developed by individual MUs with mean force values for S, FR, and FF units (see Fig. 2.7; Fig. 4 in Luff et al. 1988). In their Fig. 4, a single FR unit with a force of 4.2 N, for example, was said to have increased in size by 18 because it was 18 times larger than the mean force of FR units in unoperated muscles. However, there is as much as a 10-fold range in FR unit force in normal muscles and an even greater range in reinnervated muscles (Gordon et al. 1988). Thus, without knowing where the single MU is placed in the population, the comparison between single and mean unit force may not be valid. The error is simply demonstrated by comparing one FR unit in our sample where a 2.6 N tetanic force was 10.5 times larger than the normal mean value of 0.22 N (shown by a * in Fig. 2.7C). However, when the MU is placed within the force distribution for the MU sample and the population response is compared with normal, it is clear that all MUs increase similarly in size (Fig. 2.3C and F) and that the mean force does not increase beyond 5 times normal (Fig. 2.5A).

All soleus MUs increased in size by 4-5 times in extensively denervated muscles ($\geq$90%), which is only half the unit expansion necessary to reinnervate all the muscle fibers. Consequently, soleus muscles did not recover fully; on average, they developed 41% of the force of the contralateral muscle, consistent with the data of Luff et al. (1988) but not with data from their later study (Luff and Torkko 1990). A striking difference of the later study was that regenerating nerves from the cut VRs contributed up to 70% of the innervation of the partially denervated soleus, which, together with the substantial soleus muscle hypertrophy, is likely to account for the more complete recovery of the muscle. There was no evidence for reinnervation of regenerating fibers or hypertrophy in the present study.

Thus our data show that there is no obvious difference between unit types, or muscles, in the extent of MU enlargement by sprouting. All MUs appear to increase in size by the same factor, which is dependent on the extent of PD (see below). When the PD exceeds 85%, the limited increase in size of 4-6 times prevents full recovery of muscle force, unless regeneration of cut nerves occurs.

2.4.2 Proportional enlargement of MUs

Force in all MUs increased in direct proportion to the extent of PD, indicating that all MUs participate in collateral sprouting, with the relative increase in unit size being the same (Fig. 2.3). For example, a MU of 20 mN tetanic force will increase by a factor of 5 for an 80% reduction in the innervation to 100 mN. A MU of 200 mN, which is 10 times larger, will increase to 1000 mN, an addition of 800 mN as opposed to 80 mN for the smaller MU. The proportional shift of the entire unit force distribution and the normal size relationships between unit force and unit axon potential amplitude in the partially denervated muscles (Fig. 2.12) provide strong evidence to show that the larger motor nerves sprout more terminals than the smaller nerves. Thus enlargement of MU size by collateral sprouting maintains the normal size relationship among IR, unit force, and motor nerve size (Gordon et al. 1991; Henneman and Mendell 1981; Tötösy de Zepetnek et al. 1992), extending previous findings that regenerating nerves undergo size-dependent branching to reinnervate denervated muscle fibers after complete nerve transection (Gordon and Stein 1982b; Tötösy de Zepetnek et al. 1992). Motoneuron size or properties associated with size therefore appear to determine the final number of muscle fibers that the motoneuron supplies (IR) during reinnervation of both fully and partially denervated muscles.

2.4.3 Activity and MU size

During both sprouting in partially denervated muscles and reinnervation of denervated muscles, the larger motor nerves which are presumably the least active since they are recruited less frequently during normal muscle contraction (Henneman and Mendell 1981), capture the largest number of available muscle fibers (Fig. 2.3; see also Discussion in Callaway et al. 1989). The neuromuscular junction is therefore not a Hebbian synapse because the less active neurons are at an advantage in maintaining the highest number of nerve terminals and innervate the largest number of target muscle fibers, and not the converse as suggested by competition experiments of Ribchester and

Taxt (1983). The frequent findings that S units generate more force than normal after reinnervation (Foehring et al. 1986; Gordon and Stein 1982b) or cross-reinnervation (Gordon et al. 1988) is also unlikely to arise from the suggested competitive advantage of active MUs. The increase in force is more likely due to the larger range and mean size of SO fibers in reinnervated muscles (Gordon and Stein 1982b). The size range of SO fibers also increased progressively with the extent of PD (Figs. 2.8 and 2.9; Table 2.3). The increased size of SO fibers and trend for FG fibers to decrease in size accounted for the slightly larger than predicted increase in force of S units and less-than-predicted increase in FF units in partially denervated muscles (Fig. 2.7). Thus measurements of unit force probably overestimate and underestimate the sprouting capacity of motoneurons that supply S and FF units, respectively. Nevertheless, differences in force of S < FR < FF remained in the same order (Fig. 2.7), in agreement with that observed in normal and reinnervated muscles (Stein et al. 1990, Tötösy de Zepetnek et al. 1992).

2.4.3.1 Enlargement of neonatal units

Size-dependent branching was the same for motoneurons that sprouted in the kitten and the adult (Fig. 2.5), confirming results of Fisher et al. (1989) that neonatal units that share innervation with other units (polyneuronal innervation), can retain their excess terminals and sprout further after PD. Neonatal and adult motoneurons therefore enlarge their MUs in direct proportion to their size and can supply all denervated fibers for PDs of up to 85%. The increase in unit size by sprouting is less for the neonatal units, which simply retain their excess terminals. The net result is that the adult unit in the partially denervated muscle, whether unit size was initially larger than normal or not, is enlarged to the same extent.

2.4.4 Size of motor nerves

The finding that differences in motor axon size are established later than the differences in MU size may suggest that the size of the terminal innervation field influences motor axon size (Theriault and Tatton 1989; Vejsada et al. 1985). However, nerve fiber size was not altered by large increases in MU size, either in the neonate or adult (see also Brown and Ironton 1978; Luff et al. 1988; Luff and Torkko 1990), which argues that any retrograde influence on motor nerve caliber is relatively small or that it rapidly declines after birth. In contrast, there are significant retrograde effects on soma size and electrical properties: soma size increased significantly after PD (Tissenbaum and Parry 1991) and duration of the afterhyperpolarization of antidromic action potentials was reduced (Foehring et al. 1986; Huizar et al. 1977).

2.4.5 Reinnervation by regenerating nerves

In the light of the well-established paucity of muscle reinnervation when nerves are lesioned at great distances from the denervated muscle (Sunderland 1978), it was surprising that cut spinal roots regenerated remarkably well in some instances. Interestingly, these regenerating nerves succeeded in making functional connections only in extensively denervated gastrocnemius muscles. All muscle fibers in muscles partially denervated by 70-75% are reinnervated by terminal sprouts from the remaining intact nerves. As a result, regenerating nerves must displace terminal sprouts to account for the finding that they supply up to 50% of the muscle reinnervation (Table 2.2). For more extensive denervations (>80%), the remaining 2-15% of motor nerves enlarge to a limit of 4-6 times, but still fail to supply all denervated muscle fibers. It is, therefore, likely that regenerating nerves reinnervate the available denervated muscle fibers to account for up to 80% of the total muscle innervation (Table 2.2).

These data are consistent with previous findings that regenerating nerves can displace nerve terminals supplied by collateral sprouts (Bennett and Raftos 1977; Brown and Ironton 1978; Grinnell and Herrera 1981; Ribchester and Taxt 1984; Thompson 1978) and they explain the findings of Westerman et al. (1979) that the size of muscle supplied by nerve in the uncut VR of partially denervated cat FDL muscles appear to decline with time. The finding that displacement of terminals occurs only when there are large increases in the size of the muscle unit is consistent with previous findings in frog muscle, where displacement occurs most readily when synaptic efficacy of the terminals is low (Werle and Herrera 1987). Failure of extensively enlarged motor units to maintain tetanic force at high frequencies (Luff and Torkko 1990) is consistent with findings that synaptic efficacy of the terminals declines in very large units (Rochel and Robbins 1988) and that terminals with lower efficacy are displaced most readily (Slack and Hopkins 1982). Furthermore, it has been postulated that terminals with lower synaptic efficacy are also more readily withdrawn during neonatal development to establish the single innervation of each muscle fiber (Bixby et al. 1980).

Our finding that regenerating nerves did not make functional connections with partially denervated soleus muscles, in contrast to findings of Luff and Torkko (1990), may be due simply to the choice of root sectioned: S_1 (present study) vs L_7 (Luff and Torkko 1990). Section of S_1 spinal root results in extensive denervation of LG muscle (>85%), which, in this study, was readily reinnervated by regenerating nerves. As a result, few regenerating nerves remained to supply denervated soleus muscle fibers. Section of L_7 VR (Luff and Torkko 1990), on the other hand, may result in more moderate PD (<85%) of LG muscles. Consequently, the sprouts in LG will not be displaced and the regenerating nerves can grow to the more distal soleus to innervate denervated muscle fibers.

Taken together, this evidence suggests that displacement of terminals is not simply a function of time (Brown and Ironton 1978; Ribchester and Taxt 1984) or age (Table 2.2; Fisher et al. 1989), but rather a function of size of the remaining enlarged MUs. Displacement of terminals with low synaptic efficacy in neonatal and PD muscles suggests that connectivity is relatively stable for small expansions of MU size (2-4 times); but, once synaptic efficacy of terminals is compromised by excessive branching of motoneurons, the excess terminals are readily displaced. The net result is that both intact and regenerating nerves can enlarge their MUs to supply a larger number of muscle fibers (Fig. 2.13). Although large MUs are functional for long periods of time, it is clear that they are less stable than normal when they are more than 4-6 times their normal size.

2.5 CONCLUSIONS

The results of this study of MUs in partially denervated neonatal and adult muscles show that motoneurons increase the size of their MUs in proportion to their size to compensate for PDs that remove up to 85% of the normal innervation. This size-dependent branching increases MU size by up to 4-6 times. Although MUs are stable at 2-3 times their normal size the relative ease of displacement of terminals in MUs that exceed their normal size by 3-6 times suggests that there is an upper limit to the number of functional nerve terminals that a motoneuron can support.

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3. REINNERVATED UNIT FORCE

3.1. INTRODUCTION

Functional recovery after peripheral nerve injury and surgical repair depends on the accuracy of connections between regenerating motor nerves with their original muscles, the number of regenerating nerves that make functional neuromuscular connections, and how many muscle fibers become reinnervated. Thus, the prerequisites for recovery are accurate reinnervation and restoration of the normal number and size of motor units (MUs; Gordon et al. 1990). Accuracy of muscle reinnervation depends on the type of nerve injury and distance of the lesion from the target muscle. Following a crush injury the continuity of the basal lamina remains. As a result, the accuracy is generally excellent (Swett et al. 1990) and muscle recovery good (Sunderland 1978) since regenerating axons elongate within their original distal parent endoneurial Shwann tube to the correct muscles. Observations that reinnervated muscles show the normal mosaic distribution of fiber types suggests that regenerating nerves even find their original muscle fibers within the correct muscle (Haftek and Thomas 1968). When nerves are completely sectioned and surgically repaired by apposing proximal and distal stumps (nerve-nerve suture; N-N) the regenerating axons show little specificity for their original target muscles (Bernstein and Guth 1961; Miledi and Stefani 1969; Sperry 1941; Thomas et al. 1987; Weis and Hoag 1946), presumably because the axons have entered incorrect parent endoneurial tubes that guide them to inappropriate muscles. Even if the nerve is sectioned close to the muscle, and the muscle is self-reinnervated, the loss of the normal mosaic indicates that nerves do not reinnervate their former muscle fibers (Kugelberg et al. 1970).

The number of axons that reinnervate muscle fibers depends on the nature of nerve injury and method of surgical repair, age, level of injury and the delay in nerve repair (reviewed Terzis and Smith 1990). Recovery of muscle force after nerve crush is believed to be greater than after complete nerve transection and N-N repair because nearly all axons regenerate beyond the site of the nerve crush (Swett et al. 1991), while fewer axons regenerate beyond the scar tissue of the suture line (Gutmann and Sanders 1943; Gordon and Stein 1982a). The number of axons that form functional connections is even smaller when the proximal nerve stump is sutured directly to the denervated muscle (nerve-muscle; N-M suture, Gillepsie et al. 1986; Gordon and Stein, 1982ab; Fu and Gordon, 1991).

The size of the MU depends on how many nerves reinnervate the muscle and whether, under conditions where many regenerating motor nerves fail to make functional connections, the successful nerves enlarge to reinnervate all available denervated muscle fibers. The ability of MUs in partially denervated muscle to increase in size by extending nodal and/or terminal sprouts is well recognized (reviewed by Brown et al. 1981). All partially denervated muscles recover completely so long as 15 to 20% of the normal complement of MUs remain (Chapter 2; Brown and Ironton 1978; Fisher et al. 1989; Gorio et al. 1983; Thompson and Jansen 1977; Yang et al. 1990). With less innervation, the few remaining MUs cannot compensate for the lost innervation due to a limit in their sprouting capacity.

Abnormally large electromyogram (EMG) potentials in reinnervated human muscles have been interpreted as evidence for enlarged MUs to compensate for reduced MU numbers (Erminio et al. 1959; Rainbault 1987; Yahr et al. 1950). Unit force recordings in human (Thomas et al. 1987) and animal muscles after N-N suture show that the number and size of reinnervated MUs are often very similar to normal and the reinnervated muscles recover very well (Albani et al. 1988; Chan et al. 1982; Foehring et al. 1986a; Gordon and Stein 1982ab; Tötösy de Zepetnek et al. 1992; however see Bagust and Lewis 1974). However, the clinically relevant question, which has not yet been answered, is whether regenerating nerves can form enlarged MUs when the number of reinnervated MUs is reduced thus mimicking the clinical condition after severe nerve injuries. In animal experiments, where the cut nerves were sutured directly to denervated muscles (N-M suture), unit force was not significantly greater even though the number of MUs was reduced by as much as 50%. Many muscle fibers remained denervated and the muscles failed to fully recover from the denervation (Gillespie et al. 1986; Gordon and Stein 1982ab). These results suggest that regenerating nerves do not have the same capacity as intact nerves to enlarge their MUs. However the experiments do not distinguish whether the apparent inability to enlarge MUs is related to regeneration per se or to the method of surgical repair (ie. N-M suture).

In this study we have systematically reduced the number of reinnervated MUs independently of the type of nerve injury and surgical repair to ask three questions: 1) can regenerating nerves expand their MU size under conditions where the number of functional MUs is reduced, 2) does the type of nerve injury and repair influence the capacity of regenerating nerves to form enlarged MUs, and 3) do all motoneurons, small and large, have the same capacity to form enlarged MUs as shown in partially denervated muscles (Chapter 2). MUs in a partially denervated muscle increased in size by the same factor and force remained well correlated with nerve size. These findings argued that MU enlargement is size dependent with larger motoneurons supplying more sprouts than smaller motor axons (Chapter 2). Although force and axon size are well correlated after reinnervation (Gordon and Stein 1982b; Gordon et al. 1986b), several authors have noted that the smaller, slow MUs are consistently larger than normal (Desypris and Parry 1990; Foehring et al. 1986a; Gordon and Stein 1982b; Tötösy de Zepetnek et al., 1992ab).

Some results have previously been published in abstract form (Rafuse et al. 1989).

3.2 METHODS

A total of 36 cats (17 females, 19 males), with a mean weight (± S.E.) of 3.1 ± 0.1 kg (range: 2.5-4.2 kg), were used in this study. Eight of these cats were unoperated, control animals. Twenty-eight cats received the initial surgery between 3.0-16 months (mean ± S.E.: 7.6 ± 0.83 months) prior to the final acute experiment. Of the 28 experimental animals, MUs were isolated and characterized in 25 cats. In all cats the left unoperated medial gastrocnemius (MG) muscle served as a control for the right experimental MG muscle. Since Chapters 3-5 will be submitted together for publication as companion papers, and since the previous chapter has already been published, some of the methods appearing in this Chapter have been discussed in Chapter 2.

3.2.1 Initial Surgery

All cats were administered subcutaneous injections of antibiotic (ampicillin: 10 mg/kg) one hour prior to surgery to reduce risk of infection. No operated animals became infected as a result of the surgery performed. Cats were anesthetized with intraperitoneal (IP) injection of sodium pentobarbital (Somnotol: 40 mg/kg). To ensure that the cats remain anesthetized at a depth sufficient to maintain aflexia throughout the surgical procedure, some animals were also administered halothane. Under strict aseptic conditions approximately 30 mm of the MG nerve was surgically isolated within the popliteal fossa of the right hindlimb in order that the MG muscle could be denervated by either crushing or sectioning the MG nerve. In 7 cats the MG nerve was crushed with small surgical forceps at a distance of either 1 or 30 mm from the entry point of the nerve into the MG muscle. Since no significant difference in unit force (or other unit properties) was found between reinnervated muscles with crush injuries at 1 or 30 mm distances the 7 cats are considered as one group and referred to only as nerve crush throughout the paper. In 11 cats, the MG nerve was cut approximately 10 mm from its entry into the muscle and the proximal end joined to its distal stump (N-N suture) with two 8-0 silk sutures through the epineurium. In 10 cats, the MG nerve was sectioned and the proximal end secured with two 8-0 sutures to the MG muscle fascia (N-M suture) approximately 10 mm rostral to the original entry point of the nerve into the muscle. The remnants of the distal stump was carefully excised after N-M suture.

To reduce the number of regenerating motor nerves either the right L7 (4 cats) or S1 (24 cats) spinal root was cut extradurally by performing a small laminectomy between the S1 and L7 vertebrae to expose the dorsal and ventral roots. No precautions were taken to prevent regeneration of axons from the cut spinal root. Following surgery the cats were administered ampicillin (10 mg/kg) as well as an analgesic (bupromorphine: 0.01 mg/kg) to decrease any discomfort associated with the surgery. Cats were housed in large cages which permitted normal walking and playful activities.

3.2.2 Preparation for acute experiment

Acute experiments were performed under deep anesthesia by IP injection of Somnotol (40 mg/kg). The trachea was cannulated to permit artificial respiration if required. The cephalic vein in one forelimb was cannulated for administration of additional injections of Somnotol (1.0% initial dose) to maintain a level of anesthesia where withdrawal and eye-blink reflexes were suppressed. A laminectomy was performed between S2 and L5 vertebrae to expose the S1 and L7 spinal roots. Muscles in the hips and hindlimbs on both sides of the animal were denervated by cutting or crushing all nerves except for the MG nerve. The MG muscle in both the left unoperated leg and the right operated leg were exposed and freed from connective tissue. Great care was taken to ensure that the blood supply to the muscles was not compromised. The distal MG tendon was freed along with a small piece of calcaneus bone and securely tied with silk thread (#5) for later attachment to a force transducer. The MG nerves were carefully dissected from the popliteal fossa in order that a small length (~20 mm) could later be placed over an array of 5 electrodes used to either stimulate the nerve or record extracellular axon potentials. A bipolar pad electrode was sewn to the MG muscle fascia along its midbelly for recording EMG potentials.

The cats were mounted in a stereotaxic frame with pins securing the head, hips, knees and ankles. Skin flaps around the spinal cord, left and right hindlimbs were extended to form 3 pools that were filled with human grade mineral oil. The silk threads fastened to the distal tendons of the MG muscles were tied to a force transducer for recording whole muscle (Grass FT10) and unit force (Kulite transducer). Ventral roots S1 and L7 were isolated by longitudinally cutting the dura mater and cutting the roots as they entered the spinal cord at approximately the level of the L5 vertebra. The temperature of the rectum and all 3 pools was monitored throughout the experiment and maintained at 37° and 34°C, respectively, with the use of radiant heat above and a heating pad below the animal. The slightly lower than normal temperature was selected as we have consistently observed that muscles are better maintained at 34°C for the 24 hour duration of the experiment. Muscles developed as much force at the end of the experiment as at the beginning.

3.2.3 Quantification of reinnervated MU number

The number of motor nerves reinnervating the MG muscle varied considerable between animals because 1) the relative contributions of S1 and L7 ventral roots to the motor innervation of the MG muscle varies considerably between different cats and 2) the capacity for motoneurons to regenerate beyond the suture site depends on the type of nerve injury and quality of repair (Gutmann and Sanders 1943; Sunderland, 1978). The number of MUs in the reinnervated MG muscle supplied by the uncut ventral root was determined by dividing the maximum MG muscle tetanic force (elicited upon stimulation of the uncut ventral root) by the mean unit tetanic force (calculated from a large sample of MUs equalling 15-100% of the total MU number). Dividing maximum whole muscle force by mean unit force has previously been shown to be a reliable technique for calculating MU number in animal (Jansen and Fladby 1990) and human muscles (Stein and Yang 1990).

The proportion of reinnervated MUs (% MU) was calculated to be:

$$\%\ MU = (MU_n/280) \times 100\%$$

where MU_n is the estimated number of reinnervated MUs supplied by the uncut ventral root and 280 is the number of motor axons normally innervating the cat MG muscle (Boyd and Davey 1968). Since no precautions were undertaken to prevent regeneration of axons in the cut spinal root, some MG muscles ($n=15$) were reinnervated by a variable number of motor axons in the sectioned root. As found previously, cut ventral roots are surprisingly successful at regeneration (Chapter 2; Luff and Torkko 1990). The number of reinnervated MUs supplied by the cut ventral root was estimated by the same method used to calculate the amount of reinnervation by motoneurons in the uncut ventral root and the total % MU is used throughout the paper.

3.2.4 Whole muscle force and unit recordings

Twitch and tetanic force of the left and right MG muscles was measured in response to stimulation of the MG nerve with a single pulse or a train of 21 pulses at 100 Hz, respectively. Comparison of whole MG muscle force in reinnervated and contralateral normal MG muscles was used to assess the extent of recovery. The mean (± S.E.) tetanic force (62 ± 2.3, $n=28$) of the unoperated MG muscles was not significantly different (student t-test, $p<0.01$) from the mean (± S.E.) tetanic force (68 ± 5.8) of the 8 age and weight matched control cats. This similarity indicates that the force of the contralateral muscles did not change significantly as a result of the MG muscle denervation and spinal root section on the experimental side and was therefore a valid control for comparison.

Single MUs in the experimental muscle were isolated by splitting either S1 or L7 ventral roots until a single functional motor axon to the MG muscle was identified. The criteria for identifying a single MU were: 1) an all-or-none single extracellular axon potential recorded on the MG nerve, 2) an all-or-none EMG signal and 3) a all-or-none twitch response recorded at threshold voltage. The extracellular axon potential was recorded from the MG nerve which was mounted on an array of 5 silver electrodes. The electrode array was used in a triphasic configuration to record extracellular axon potentials differentially between the center and nearest 2 electrodes with the 2 outermost electrodes grounded to reduce contamination of the axon potential recording by the EMG signal (Gordon et al., 1986b). All recorded signals were amplified and the EMG and axon potentials were filtered with a 60 Hz notch filter if required. Between 10 and 40 stimulus responses were averaged using a PDP 11/21 microcomputer and stored on disk for further analysis. All signals were monitored throughout the experiment on a storage oscilloscope (Tektronix 5441) and the force and EMG signals on a Gould chart recorder.

Ten to 40 responses were averaged to the following regimes: 1) one pulse, 2) 21 pulses with an interpulse interval of 10 ms, 3) one pulse immediately following 6 tetanic responses, 4) a train of pulses at an interval of 1.25x twitch contraction time for a duration of 800 ms, 5) 13 pulses with an interpulse duration of 25 ms every second for 2 minutes, and 6) one pulse. These stimulus regimes were used for each MU to record: 1) twitch force, contraction time and 1/2 fall time, 2) tetanic force, 3) potentiated twitch contractions, 4) presence or absence of sag in an unfused tetanus, 5) fatigue index measured as a ratio of last contractions to the first in a 2 minute fatigue test, 6) post-fatigue twitch contraction. Axon size was estimated electrophysiologically by recording the extracellular axon potential amplitude measured during each twitch response and by the axon conduction velocity. Axon potential amplitude (μV) was normalized by the mean (± S.E.) nerve-electrode contact impedance (6.1 ± 2.8 kohms) measured from all controls experiments in order that the potentials could be compared from animal to animal (see Gordon and Stein 1982ab). Conduction velocity was determined by dividing the distance (mm) between the ventral root filament stimulating and MG nerve recording electrodes by the latency (ms) of the positive peak of the axon potential.

MUs were classified as slow (S), fast-fatigue resistant (FR), fast-fatigue intermediate (FI), and fast-fatigable (FF) according to their twitch contraction time or sag characteristic as well as their fatigue indices (Burke et al 1973; Fleshman et al 1981; Gordon and Stein 1982a). Fast MUs had a twitch contraction time $\leq$40 ms while slow MUs had a contraction >40 ms. FF units had a fatigue index <0.25, FI units had a fatigue index $\geq$0.25 and $\leq$0.75 while FR and S units had fatigue indices >0.75. MUs with a contraction time between 35 to 45 ms were classified on the basis of sag (ie. F units sag, S units do not).

3.2.5 Glycogen-depletion protocol

To ensure that only muscle fibers belonging to the MU selected for glycogen-depletion were depleted of glycogen, and to increase the contrast between the depleted and nondepleted muscles fibers, all cats were glucose loaded with the use of 5% glucose in the drinking water 3 to 4 days prior to the final acute experiment.

After characterizing a large sample of MUs (9 to 56), one unit was stimulated repetitively to deplete its muscle fibers of glycogen. The depleted muscle fibers were later visualized histochemically. At least 1 hour was allowed to pass after the last MU was characterized prior to the onset of the stimulation paradigm to glycogen-deplete the muscle fibers of the single MU. The stimulation protocol used was a modification from Tötösy de Zepetnek et al. (1992a). Briefly, the general location in the muscle of the selected MU was visualized upon stimulation of the ventral root filament. The MU was stimulated by a tetanic train of 10 pulses at 40 Hz every second. The interval between trains of pulses was gradually decreased from 1 second to 0.5 seconds until MU fatigue was induced. Care was taken to ensure that the amplitude of the EMG signal did not decrease by more than 20%. If the EMG signal decreased too rapidly, either the number of pulses in the stimulus train or the repetition rate was reduced to prevent neuromuscular fatigue, and in turn, to prevent incomplete glycogen-depletion of all muscle fibers belonging to the MU (Barker et al. 1977). Once unit force decreased to approximately 10% of its original value the force of F units was allowed to recover by increasing the interval between trains of pulses from 0.5 to 2 seconds. MUs were stimulated for 2 to 5 minutes at this rate after which the cycling time between trains of pulses was again slowly decreased to 0.5 seconds. This glycogen-depletion protocol was repeated between 3 to 7 times for F units, but only once for S units. S units required up to 3 hours of continuous stimulation before the force decreased to 10% its original value.

Immediately after cessation of the glycogen-depletion paradigm the MG muscles in both hindlimbs were quickly excised, weighed and placed in cooled saline. All animals were euthanized with an overdose of Somnotol. The muscles were cut into 5 blocks along the muscles proximodistal (longitudinal) axis, fixed to a piece of cork with

OCT (ornithine carbamyl-transferase), frozen in a pool of melted isopentane cooled in liquid nitrogen and immediately placed in a freezer (-70°C) for storage.

3.2.6 Muscle histochemistry and fiber measurements

Serial cross-sections, 10 μm thick, were cut from each muscle block and stained for myofibrillar ATPase with acid preincubation modified from Brooke and Kaiser (1970) and with alkaline preincubation modified from Guth and Samaha (1970) as described in detail by Gordon et al. (1988). Glycogen-depleted muscle fibers were identified as negatively stained fibers using the Periodic acid-Schiff (PAS) stain (Pearse 1968). The nomenclature used for the histochemically identified muscle fibers (SO: slow-oxidative, FOG: fast-oxidative glycolytic; FG: fast-glycolytic) is that of Peter et al. (1972).

Since muscle fibers in the cat MG do not extend through the entire muscle, but rather pinnate along the dorsal-lateral surfaces, a single lateral cross-section through the muscle will not section all fibers belonging to the glycogen-depleted MU (Burke and Tsairis 1973). However, if several proximodistal sections are made through the muscle the cross-section that contains the largest number of glycogen-depleted muscle unit fibers corresponds to the center of the unit territory (Burke and Tsairis 1973). If it is assumed that all MU territories have the same general shape then the distribution and number of glycogen-depleted muscle unit fibers in this cross-section should provide a relatively accurate representation of the whole MU territory, muscle unit fiber distribution and unit innervation ratio (IR; Burke and Tsairis 1973).

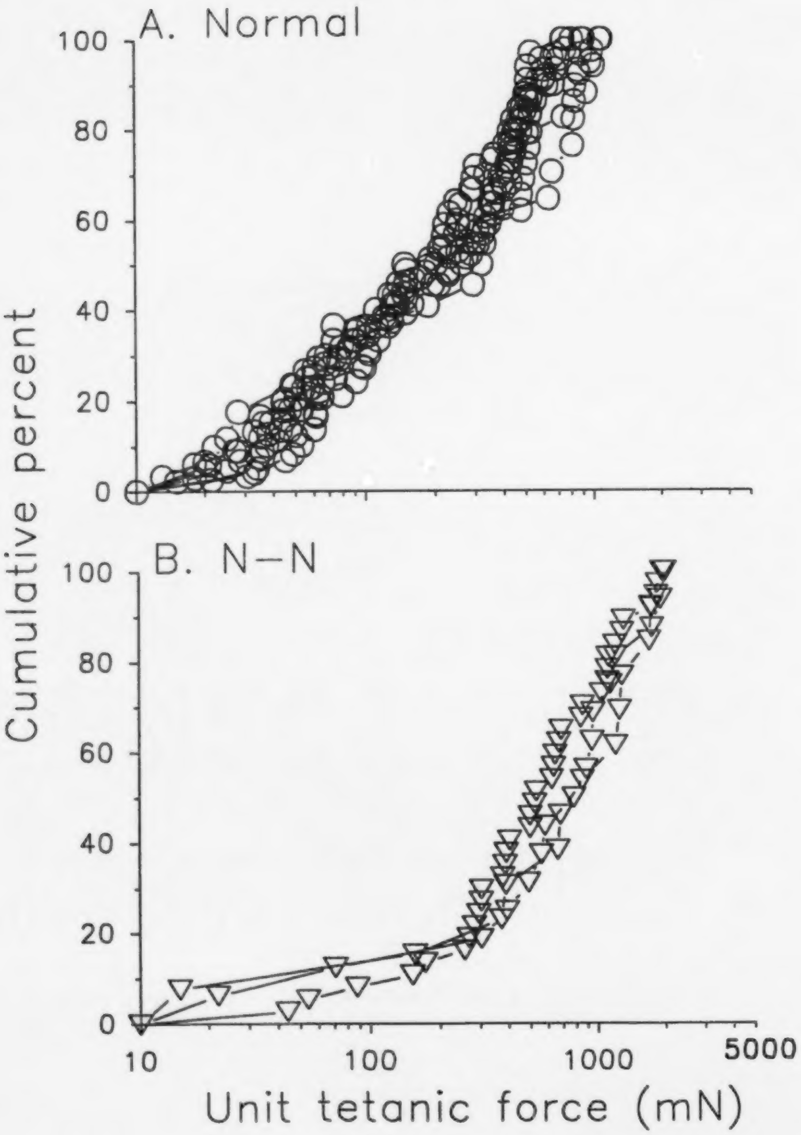
The number of glycogen-depleted muscle fibers were counted in the muscle cross-section containing the largest number of depleted fibers and the cross-sectional areas (CSAs) of 159-210 fibers were measured with a microcomputer digitizing software program (JAVA, Jandel Scientific) which was linked from the computer to a microscope via a video camera. The proportion of muscle fiber types and CSA measurements of nondepleted fibers were determined from the cross-section closest to the muscle midbelly. Since the distribution of muscle fiber types in the cat MG muscle is highly regionalized, muscle fibers were counted and measured in 4 to 6 different areas (0.796 mm^2 each) of the muscle spanning the medial to lateral regions of the muscle.

3.2.7 Statistical analysis

Fifteen to 100% of the total MU population in each MG muscle was sampled in control and experimental animals to physiologically characterize the MUs and to compare electrophysiological parameters of MU and nerve size: namely isometric force, axon potential amplitude and conduction velocity. Differences in the distribution of unit force between MG muscles were tested for using the nonparametric Kruskal-Wallis test (Sokjal and Rohlf 1969) which tests for differences in the distribution of the rank order of each parameter measured. Unit force values between different animals were pooled together only if they were not significantly different at the 0.95% confidence level. Two examples in which the distribution of unit force were not significantly different ($p > 0.95$) and therefore could be pooled together are illustrated in Fig. 3.1. Fig. 3.1A shows the unit force distribution of 8 normal MG muscles while Fig. 3.1B shows the unit force

Figure 3.1.

Cumulative distribution of tetanic force of 12 to 51 MUs in each of 8 individual normal muscles (A) and in 3 muscles reinnervated by 25 ± 4.3% of its original complement of MUs after N-N suture (B). Rank order analysis, using the Kruskal-Wallis test, showed that the force distributions plotted within (A) and (B) were not significantly different from each other ($p<0.01$), but the distributions are different between (A) and (B) ($p<0.01$).

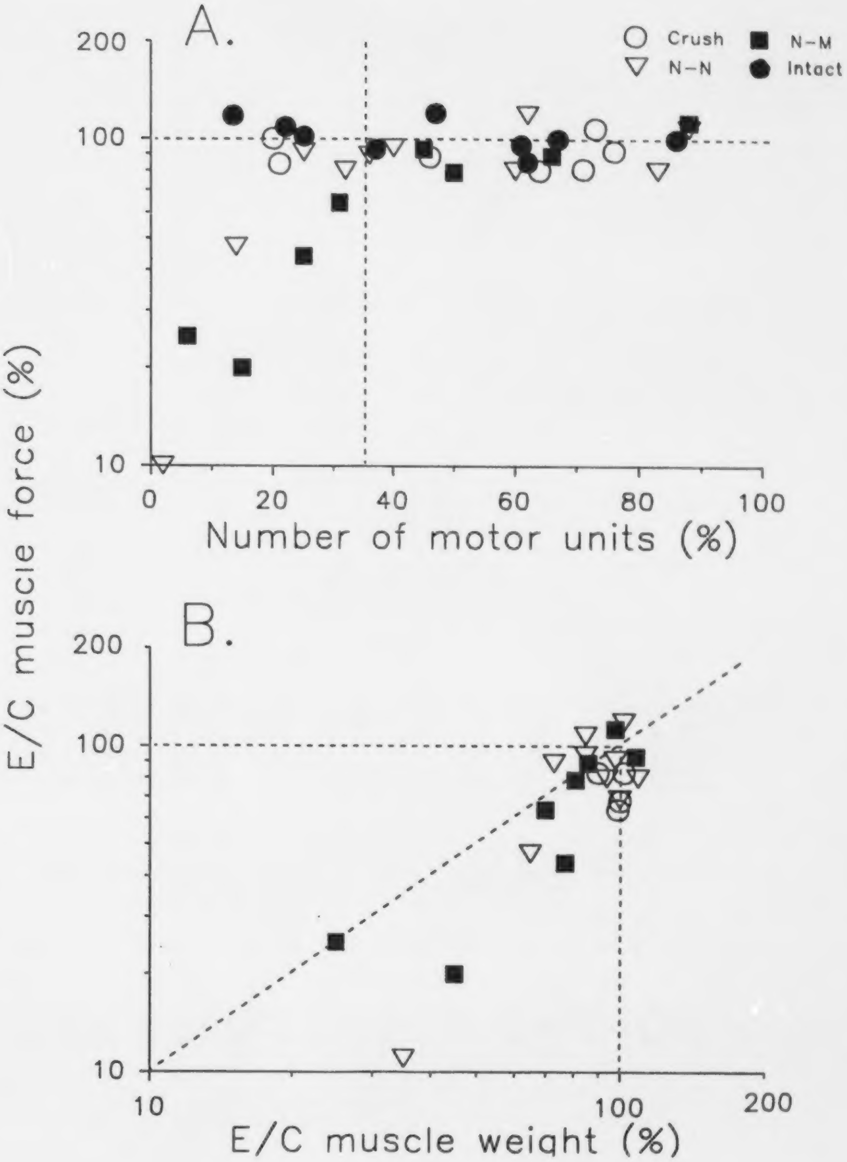

A. Normal
B. N-N
Cumulative percent
0
20
40
60
80
100
10
100
1000
5000
Unit tetanic force (mN)

distribution of 3 experimental animals in which only ~25% of the MUs reinnervated the MG muscle after N-N suture. The pooled data were collected by using logarithmic bin intervals to represent the logarithmic distribution of force values (Fig. 3.3). The force on the X-axis is shown in logarithmic intervals of equal bin width and not of the exponential values used to calculate the bin intervals in order that the actual force values can be more readily visualized (Fig. 3.9A and C). Significant differences between cumulative distributions were tested for by applying the Kolmogrov-Smirnov test (Fisz 1963).

Throughout this paper, arithmetic means are given with standard errors (S.E.). Statistical difference between 2 means was determined using the Students t-test. Regression lines were fitted according to the least mean squares criterion (Hartley 1961).

Figure 3.2.

Whole muscle force recovery, calculated as a percent of the contralateral control muscles, is plotted against the number of MUs (%) in the experimental muscle (A) and against muscle wet weight, calculated as a percent of the contralateral muscle (B). Different symbols represent partially denervated muscles (filled circles), reinnervated muscles after nerve crush (open circles), N-N suture (open inverted triangles), and N-M suture (filled squares). A greater potential for recovery of whole muscle force innervated by a few (<30%) MUs was observed in muscles following partial denervation or reinnervation after nerve crush as compared to reinnervation after N-N or N-M suture. Reinnervated muscles after N-M suture showed the poorest recovery of whole muscle force when few MUs reinnervated the muscle. Weight loss was generally greater than the reduced force in muscles that failed to recover fully (B).


A.
Crush
N-M
N-N
Intact
200
100
10
0
20
40
60
80
100
Number of motor units (%)
E/C muscle force (%)
B.
200
100
10
10
100
200
E/C muscle weight (%)

3.3 RESULTS

3.3.1 Muscle force and size

All MG muscles were reinnervated to some extent by regenerating motor nerves 3 to 16 months after either MG nerve crush, or nerve section and repair with N-N or N-M sutures. Gutmann and Sanders (1943) observed that the number of regenerating axons elongating beyond the suture line after nerve section and suture is extremely variable and that the number in the distal stump is always less than the number in the proximal stump even a year after nerve section. The number of motor nerves regenerating after crush or cut injuries was experimentally reduced further by transecting motor axons in one of two contributing ventral roots (see Methods for details). Due to the variation in both the contribution of each ventral root to the innervation of the MG muscle, and the number of axons that regenerate beyond the injury site after nerve crush or transection, the number of reinnervated MUs per muscle varied widely between experiments. In 28 experiments, the MG muscle was reinnervated by 2 to 88% of its original complement of motoneurons with a mean (± S.E.) innervation of 45 ± 5.1%. In spite of the reduced MU numbers, 16 of 25 muscles developed as much tetanic force as normal and 19 weighed the same as the contralateral control muscles.

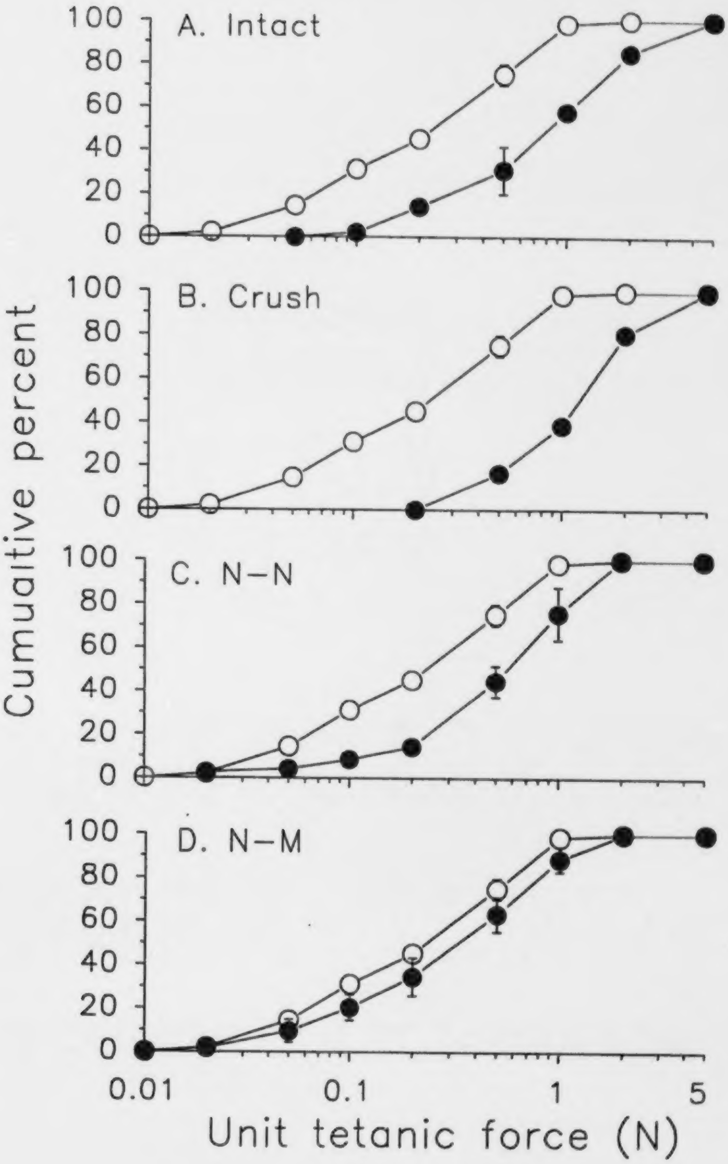
The relationship between muscle force and weight is shown in Fig. 3.2B where the two parameters are plotted, on double logarithmic axes, as ratios (%) of the contralateral control MG muscle. The tight grouping of points at 100% on both the X and Y-axes shows that most reinnervated muscles completely recover muscle weight and force. If muscle tetanic force and weight are directly related, the points representing muscles with incomplete tetanic force recovery should fall on the dashed diagonal line in Fig. 3.2B. Of the 9 muscles with incomplete recovery of tetanic force the weight loss was generally less than the reduced force presumably due to the weight of accumulated connective tissue in the reinnervated muscles. This result shows that muscle wet weight overestimates the degree of muscle recovery.

Filled circles in Fig. 3.2A show the tetanic force of partially denervated muscles, relative to the contralateral control muscles, 4 to 18 months after cutting one of the two ventral roots that contribute motoneurons to the MG muscle (data from Chapter 2). Muscles fully recovered even when only 15% of the normal complement of MUs remained. If the MG nerve was crushed and allowed to regenerate, the reinnervated muscles showed a comparable recovery of force (open circles). When motor axons regenerated after complete nerve section and N-N suture (open inverted triangles), reinnervated muscles did not recover fully when the number of reinnervated MUs was 20% or less than that of normal. When the cut nerves were sutured directly to the denervated muscles (N-M suture) (filled squares) recovery was even poorer: reinnervated muscles did not recover whole muscle force when 40% or less of the MUs did not reinnervate the muscle.

The data in Fig. 3.2A suggests that intact (nerves in partially denervated muscles) and regenerating motor nerves after crush injury, where the continuity of the endoneurial tube remains, have comparable abilities to form enlarged MUs. Severed motor nerves (N-N and N-M sutures) appear to be less able to enlarge their reinnervated MUs and

Figure 3.3.

Cumulative frequency histograms of MG unit tetanic force in normal muscles (open circles) and partially denervated muscles (Intact: A), reinnervated muscles after nerve crush (B), N-N suture (C), and N-M suture (D). The percent (± S.E.) of normal MUs in the experimental muscles was 21 ± 1.2% (n=3), 19 ± 0.71% (n=2), 24 ± 4.3% (n=3), and 24 ± 6.2% (n=5), respectively. The normal bimodal distribution of unit force is shifted to larger force values after partial denervation (A) without changing the range or shape of the distribution. The shift in unit force is comparable after nerve crush (B), but is less after N-N (C) and minimal after N-M suture (D). Small MUs increased in size more than larger MUs, after reinnervation (B,C) as shown by the greater shift to the right of force values below 50%.


A. Intact
B. Crush
C. N-N
D. N-M
Cumuaitive percent
0
20
40
60
80
100
0.01
0.1
1
5
Unit tetanic force (N)

compensate for reduced numbers of MUs.

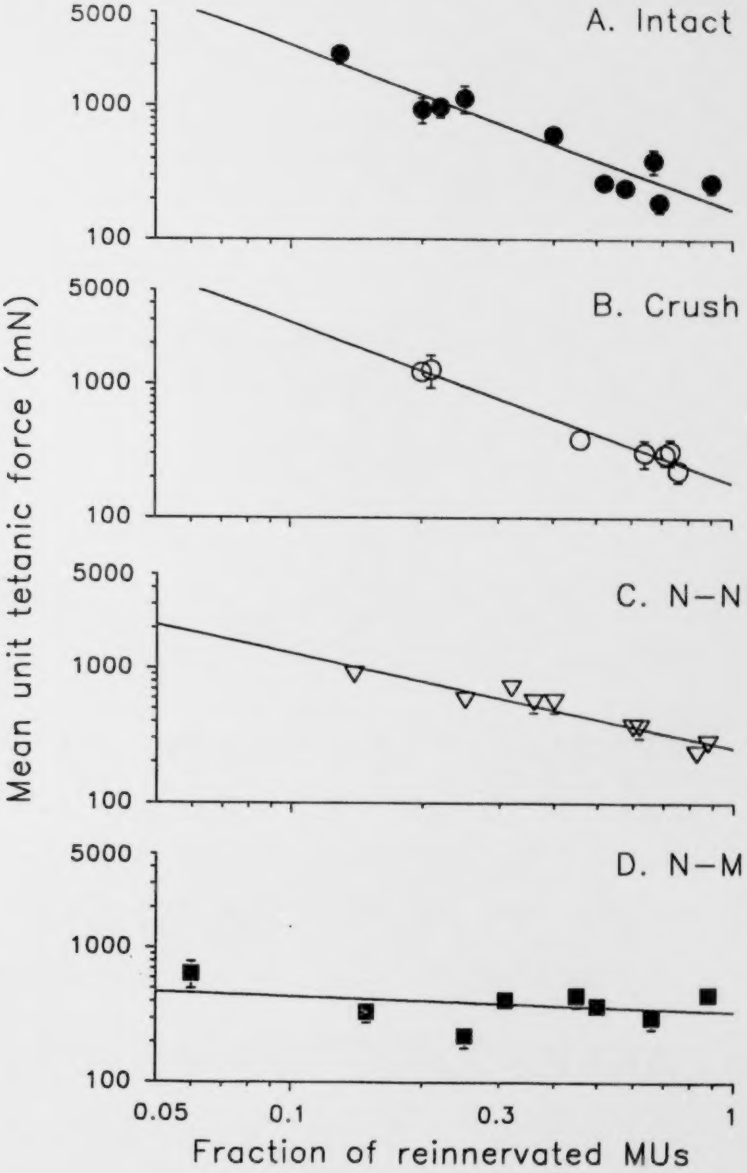
Normally, there is a 100- fold range in MG unit force as shown by the cumulative histogram in Fig. 3.3A (open symbols). The data is plotted on semi-logarithmic scales in order to compare the ability of intact and regenerating nerves to enlarge their MUs when the number of MUs is reduced to approximately 20% that of normal. MUs from 2-5 animals are included in each graph and, as described in detail in the Methods, MU contractile properties for different cats were only combined when their populations were not significantly different (Fig. 3.1). A parallel shift to the right, as shown in Fig. 3.3A for intact MUs (data from Chapter 2), indicates that all MUs increase in size by the same factor of 4 to compensate for the loss of 80% (79 ± 2, n=3) of the MU population. Regenerating nerves after crush injury have a comparable ability to increase their MU size since the reinnervated MUs enlarged by a similar extent to compensate for the loss of 80% of the MU population (Fig. 3.3B). However, when the MG nerve was cut, and the endoneurial tubes disrupted, the enlargement of MUs was smaller (Fig. 3.3C,D). In Fig. 3.3C, the number of MUs was reduced to 20% of normal, similar to that shown for intact MUs (Fig. 3.3A), however, the cumulative histogram was not shifted as far to the right (Fig. 3.3A). Thus for example, 50% of the MUs are larger than 0.8 N in partially denervated muscles as compared to 0.5 N after N-N suture; a 4-fold as compared to a 2.5- fold increase above the size of normal MUs (0.2 N) (Fig. 3.3C). When regenerating nerves are not guided by the distal nerve stump at all, as after N-M suture, the enlargement of MUs was small as indicated by the minimal shift to the right in the cumulative histograms (Fig. 3.3D). There was a noticeable trend for the small MUs to increase in size more than the large MUs after nerve regeneration which is in direct contrast to the intact nerves. This is shown by the greater shift of force values to the right below 50% than above (see Fig. 3.3B and to a lesser extent Fig. 3.3C).

The mean values of tetanic force from a MU sample equalling at least 7% of the population are plotted as a function of the number of reinnervated MUs expressed as a fraction of normal (Fig. 3.4). On double logarithmic scales, mean unit force increases as an inverse function of MU number for intact (Fig. 3.4A), crushed (Fig. 3.4B) and severed nerves with N-N sutures (Fig. 3.4C), but not after N-M repair (Fig. 3.4D). The similarity in the slope (± S.E.) of the regression lines fitted to the data for the intact and regenerating nerves after crush (-1.22 ± 0.16, RO: 0.94; -1.11 ± 0.16, RO: 0.95, respectively) (Fig. 3.4B) injuries indicates that nerves regenerating along their original endoneurial tubes have a comparable ability to enlarge their MUs as intact nerves in partially denervated muscles. The significantly lower slope of the regression line (-0.70 ± 0.09, RO: 0.95) (Fig. 3.4C) after N-N suture suggests that cut motor nerves, that do not regenerate within their original nerve sheaths (N-N suture), do not enlarge their MUs as much as those that do follow their original sheaths (nerve crush, Fig. 3.4B) (but see below). The slope of the regression line through the N-M values (Fig. 3.4D) is not significantly different from zero (0.30 ± 0.31, RO: 0.41). Thus, nerves regenerating through muscle fibers do not appear to demonstrate a significant capacity to enlarge their MUs to compensate for the reduced number.

The smallest MUs in normal muscles are generally S units as shown in Fig.

Figure 3.4.

Mean unit tetanic force (± S.E.) in partially denervated (intact; A), reinnervated muscles after nerve crush (B), N-N (C), and N-M suture (D) are plotted as a function of the fraction of reinnervated MUs on double logarithmic axes. The slope of the regression lines fitted through A and B are not significantly different from each other ($p<0.05$) indicating that crushed nerves have a comparable capacity to enlarge their MUs as intact nerves. The significantly smaller slope in C suggests that transected nerves with N-N sutures have a smaller capacity to enlarge their MUs. MUs in muscles with N-M sutures (D) did not increase in size even when few MUs reinnervated the muscle.


A. Intact
B. Crush
C. N–N
D. N–M
5000
1000
100
Mean unit tetanic force (mN)
0.05
0.1
0.3
1
Fraction of reinnervated MUs

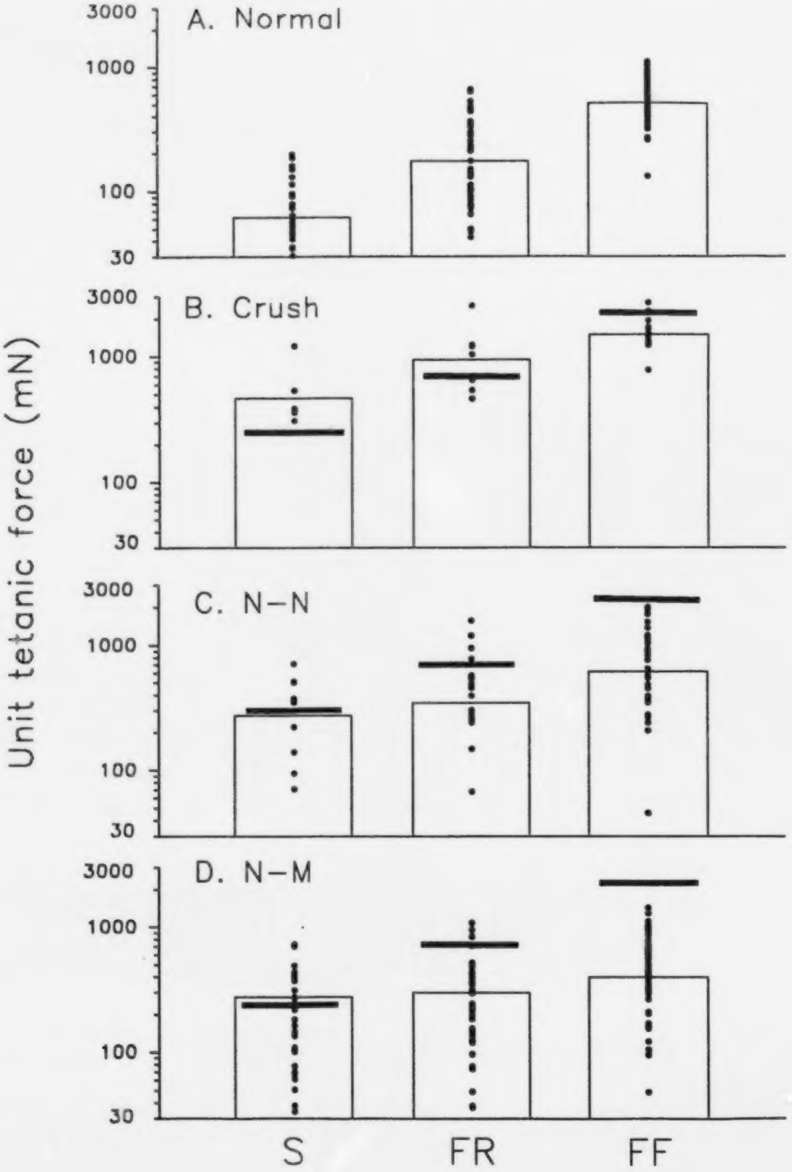
3.5A. Unit force normally increases in the order $S < FR < FF$. The data in Fig. 3.5 is for muscles reinnervated by approximately 20% of the normal number of MG units where the thick horizontal line represents the expected mean unit force if all MUs increased in size by 4-fold. The difference in force between unit types however was progressively less after crush, N-N and N-M sutures (cf. Fig. 5B-D). This occurs because S units increased more than predicted and FF units increased less. This trend for FF units becomes progressively more obvious from B to D in Fig. 3.5. After N-M suture there was little increase in size of the fast-contracting MUs (ie. FR and FF) with most of the compensation for the loss of MUs being be made by the S units. The same result was found in muscles reinnervated by more than 20% of the normal MU number. The data may be interpreted as a preferential ability of S units to reinnervate muscle fibers, but it must be remembered that unit force is also determined by the mean muscle unit fiber CSA. Differential change in mean CSA of different MU types after reinnervation (see below) does partly account for the observed increase and decrease in the tetanic force of S and FF units, respectively.

3.3.2 Reinnervated muscle fiber size

Comparison of the whole muscle cross-section, cut from the midbelly of normal (Fig. 3.6A), partially denervated (Fig. 3.6B) and reinnervated muscles after nerve crush (Fig. 3.6C), section and repair with N-N (Fig. 3.6D) or N-M sutures (Fig. 3.6E), shows the progressively poorer recovery and loss of muscle fibers, where the number of MUs was less than 25% that of normal. Across the muscle cross-section, nuscle fiber CSA

Figure 3.5.

Single unit (filled circles) and mean unit tetanic force (open boxes) of S, FR, and FF units in normal (A), reinnervated muscles after nerve crush (B), N-N suture (C), and N-M suture (D). Experimental MUs were sampled from the same muscles used in Fig. 3.3. Thick horizontal lines indicate expected increase in unit tetanic force if only 25% of the normal complement of MUs reinnervated the muscle. Normally unit force increases in the order S<FR<FF (A). This difference is progressively lost after crush, N-N, and N-M sutures (B-D). Geometric means (mN) for S, FR, and FF unit tetanic force are 62, 173, and 500 (A); 468, 933, and 1479 (B); 275, 347, and 617 (C); 277, 300, and 400 (D), respectively.


A. Normal
B. Crush
C. N–N
D. N–M
Unit tetanic force (mN)
3000
1000
100
30
S
FR
FF

is not uniform with CSA being largest superficially. For this reason fibers were sampled in each of five areas from the deep to superficial regions of the muscle. Note also that the majority of FG fibers are located more superficially than FOG and SO fibers which are more prevalent in deeper regions of normal as well as reinnervated muscles (Fig. 3.6A-C).

Fig. 3.7 shows that the size of different fiber types recovers to normal in muscles reinnervated after crush injury. The differences between the size of different fiber types (ie. SO<FOG<FG) are greatly diminished after nerve section and repair by N-N or N-M suture primarily because FG fibers are smaller than normal. Since SO fibers recovered to normal under all conditions of regeneration the increased S unit force shown in Fig. 3.5 is due to an increase in IR and/or change in specific force (SF). The finding that reinnervated FOG fibers are smaller on average than normal can account, at least in part, for the smaller increase in FR unit force than expected for proportional increase in IR to compensate for decreased MU number (Fig. 3.5C,D). Similarly, the significantly smaller FG fibers contributes to the smaller than expected increase in size of FF units.

The size distributions of SO, FOG and FG fibers overlap considerably as shown in the example in Fig. 3.8A (open histograms). After crush injury the size of SO,FOG,FG fibers was slightly smaller than normal. There was also more overlap between muscle fiber types (stippled histograms; Fig. 3.8A). The overlap was even more obvious after nerve section and repair (N-N suture), as the range in fiber size increased for each type (stippled histogram; Fig. 3.8B). Similar changes in the fiber size

Figure 3.6.

Cross-section of normal (A), partially denervated (B) and reinnervated muscles after nerve crush (C), transection and repair with N-N suture (D) or N-M suture (E) at low magnification. Muscles were stained for myosin ATPase with acid preincubation except in C with alkaline preincubation. All muscles are oriented such that the left is deep and right is superficial.

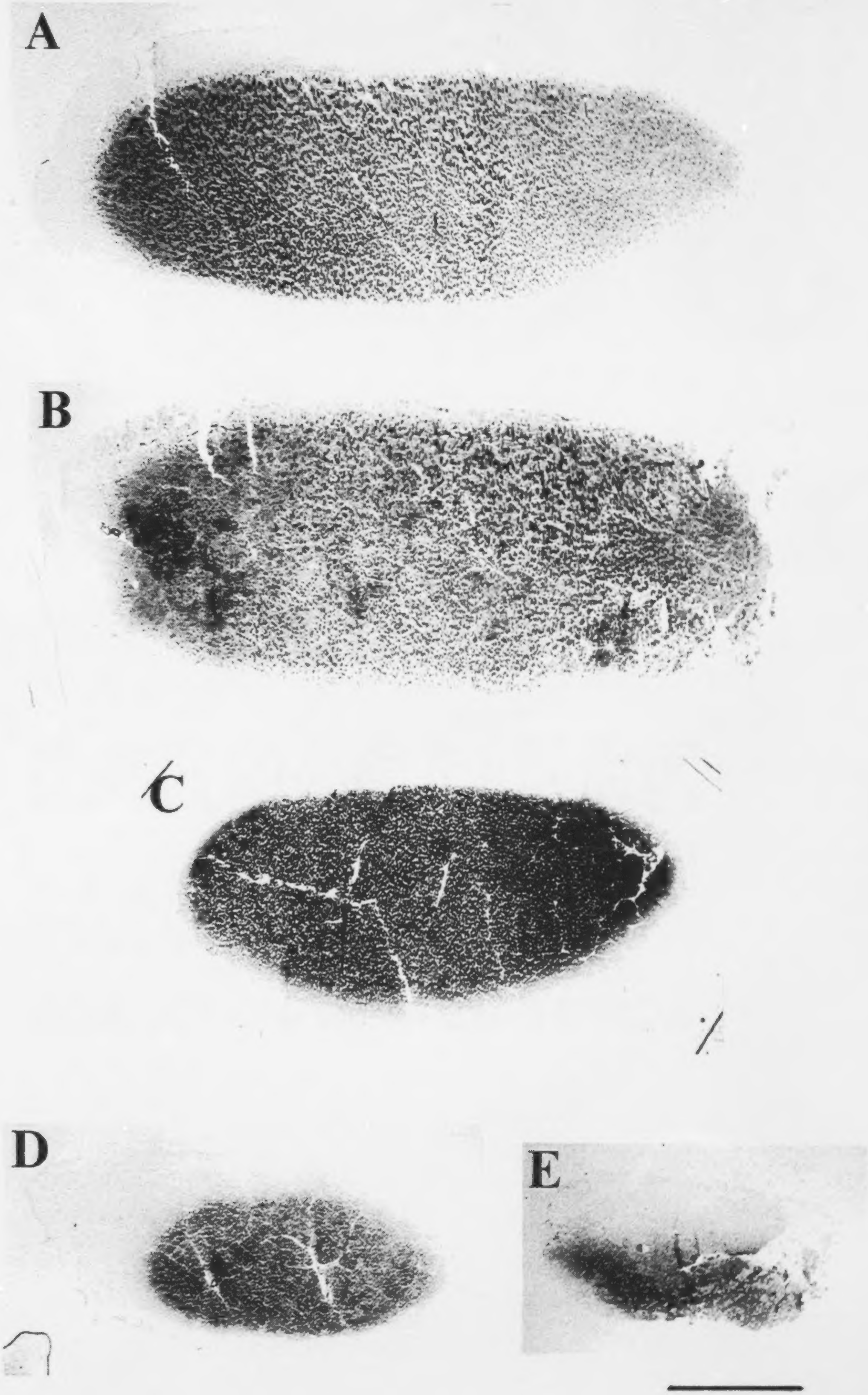
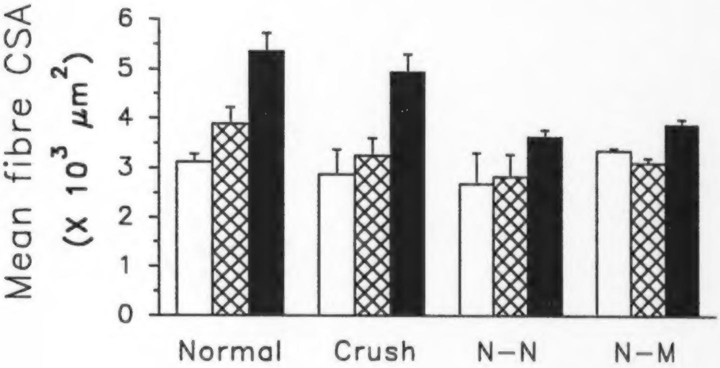

A
B
C
D
E
5 mm

Figure 3.7.

Mean (± S.E.) muscle fiber CSA of SO (open bars), FOG (cross-hatched bars), and FG (filled bars) fibers in all normal and reinnervated muscles after nerve crush, N-N, and N-M sutures. The mean size of 3 fiber types in reinnervated muscles after nerve crush are similar to normal, but FOG and FG fibers are significantly smaller than normal in reinnervated muscles after N-N or N-M suture. Mean (± S.E.) CSAs (X 10^3 μm^2) for SO, FOG, and FG fibers are 3.1 ± 0.05, 3.9 ± 0.10, and 5.4 ± 0.1 (A); 2.9 ± 0.17, 3.2 ± 0.33, and 5.0 ± 0.35 (B); 2.7 ± 0.50, 2.8 ± 0.35, and 3.6 ± 0.34 (C); 3.3 ± 0.63, 3.1 ± 0.45, and 3.9 ± 0.13 (D), respectively.


Mean fibre CSA
(X 10^3 μm^2)
6
5
4
3
2
1
0
Normal
Crush
N-N
N-M

distributions were found in reinnervated muscles after N-M suture.

Comparison of histograms of unit force and muscle fiber size of reinnervated muscles 4 (Fig. 3.9A,B; open histograms) and 11 months (Fig. 3.9A,B; filled histograms) after N-N suture, where the number of MUs was 88 and 83% that of normal, respectively, shows that the different times of observations after surgery in this study did not influence the reinnervated unit force or muscle fiber CSA. In addition, MU number did not affect recovery of muscle fiber size (Fig. 3.9D) which w a7Documentgas the same for muscles where MU number and force distributions were very different but the time after surgery was the same (4 months) (Fig. 3.9C).

3.3.3 Innervation ratio: indirect estimates

If we take muscle fiber CSA into account, unit force directly reflects IR on the assumption that SF contributes little to the variation in force between different MU types (Tötösy de Zepetnek et al. 1992a; reviewed by Stein et al. 1990). We have ted the data in Fig. 3.4, after normalizing mean unit force values by the relative size of its mean muscle fiber CSA as compared to fiber size in normal muscles, as a more direct method of comparing the increase in IR in partially denervated muscles ("intact") and after complete nerve injuries ("crush", "N-N", and "N-M") (Fig. 3.10). The slopes of the regression lines are the same for "intact" and "crush" as in Fig. 3.4., but the slope was 18% steeper for "N-N" (Fig. 3.10C) because MU size, measured as unit force, was underestimated by the reduced fiber size (Fig. 3.7). After normalization, the slopes of the regression lines were the same for "intact", "crush", and "N-N" suture conditions,

Figure 3.8.

Frequency histograms comparing the CSA of SO, FOG, and FG fibers in normal MG muscles (open histograms) with fibers in the contralateral experimental muscle (shaded histograms) which were reinnervated by 22% of the normal complement of MUs after nerve crush (A) and 36% after N-N suture (B). The mean CSAs are indicated by vertical line (normal) and vertical line with asterisks (experimental). Mean CSA values of each type are less after reinnervation, but the decrease in size is more dramatic after N-N suture. Mean CSA (X 10^3 μm^2) values for the experimental SO, FOG, and FG fibers are 3.3, 3.2 and 5.6 (A) and 2.4, 2.7, and 3.5 (B), respectively.

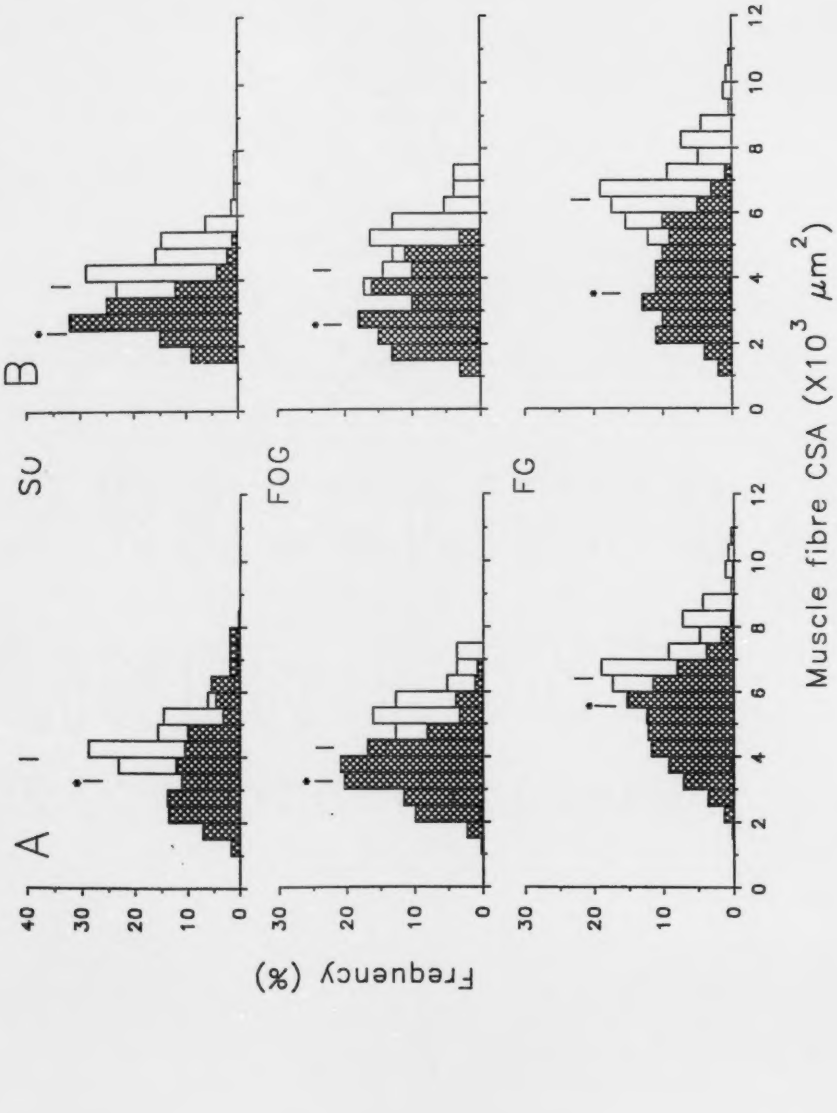
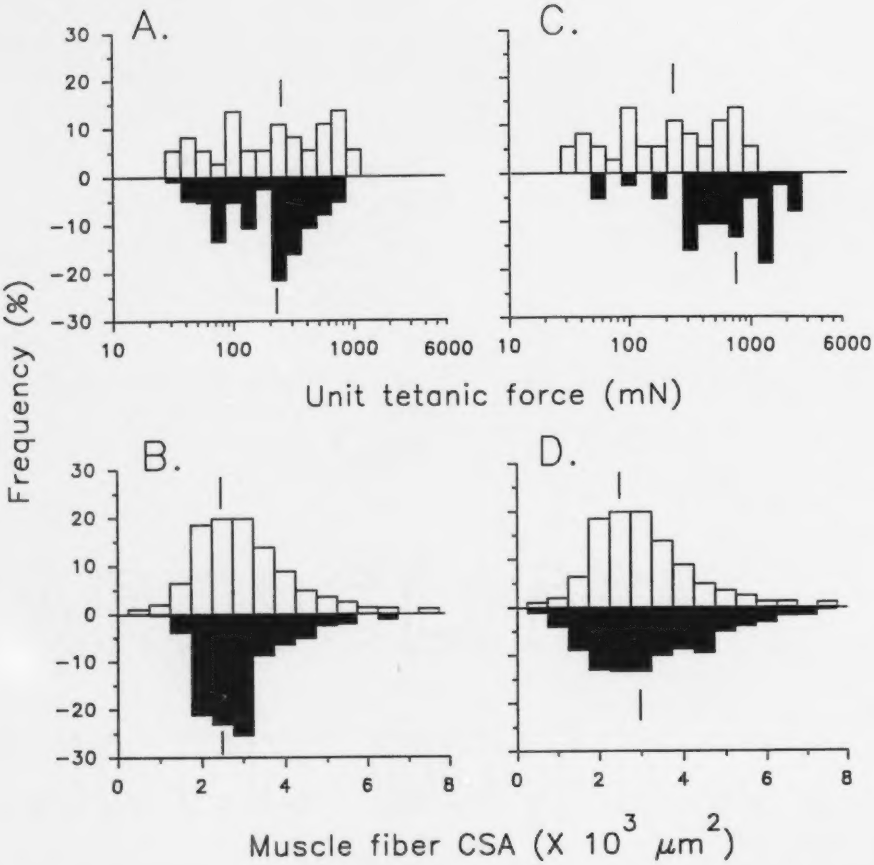

A
B
SO
FOG
FG
Frequency (%)
40
30
20
10
0
0
2
4
6
8
10
12
Muscle fibre CSA (X10^3 μm^2)

Figure 3.9.

Frequency histograms comparing the distribution of unit tetanic force and fiber CSA in muscles reinnervated by 88% and 83% of the normal number of MUs 4 (upper histograms) and 11 months (lower histograms) after N-N suture (A,B) and in muscles reinnervated by 88% (upper histograms) and 32% (lower histograms) of their normal complement of MUs 4 months after N-N suture (C,D). The different times after surgery was not a contributing factor in determining unit force (cf. A,B). The number of reinnervated MUs also did not affect the size of the muscle fibers (D) despite a large difference in unit tetanic force (C).


A.
C.
B.
D.
Frequency (%)
30
20
10
0
-10
-20
-30
10
100
1000
6000
Unit tetanic force (mN)
0
2
4
6
8
Muscle fiber CSA (X 10^3 μm^2)

showing that regenerating nerves have a similar capacity to increase in size as sprouting nerves. This capacity is compromised under conditions where regenerating nerves are forced to grow outside the endoneurial sheaths after N-M suture. Thus the methods of repair (N-M vs N-N) accounted for the reduced MU enlargement of regenerating nerves and it is not regeneration per se that accounts for the poorer recovery of whole muscle force (Fig. 3.2) and smaller increase in mean unit force (Fig. 3.4).

3.3.4 Innervation ratio: direct estimates

The most direct estimate of unit IR can be obtained by counting muscle fibers in an isolated and glycogen-depleted MU. IRs for five normal and 11 reinnervated (7 N-N, 4 N-M sutures) MUs were estimated by counting the number of glycogen-depleted muscle fibers in a single muscle cross-section (Fig. 3.11) (see Methods for details). The number of glycogen-depleted muscle fibers belonging to the normal MUs ranged from 151 to 416 fibers with a mean of 238 ± 63 fibers. The corresponding tetanic force for the normal MUs ranged from 151 to 518 mN with a mean (± S.E.) of 264 ± 61 mN. Due to the small pinnation in MG muscle fibers all glycogen-depleted fibers cannot be counted in a single cross-section. Therefore, the number of muscle unit fibers in the section containing the highest number of glycogen-depleted fibers represents only 50-67% of the total number (Burke and Tsairis 1973). Consequently, the actual mean IR is between 343 to 528 fibers.

Fig. 3.11A shows that unit force varies systematically with unit fiber in normally innervated cat MG muscles. The slope (± S.E.) of the regression line is 0.93 ± 0.23

Figure 3.10.

Unit tetanic force, normalized by mean muscle fiber CSA in normal muscles (4200 μm^2) in partially denervated (intact; A), reinnervated muscles after nerve crush (B), N-N (C), and N-M sutures (D) are plotted as in Fig. 3.4. The slope of the regression lines are not significantly different from the corresponding regression lines in Fig. 3.4 ($p<0.05$) although the slope in C increased by 18%. The slopes (± S.E.) of the regression lines fitted to the values in A,B,C, and D are: -1.20 ± 0.18 (RO: 0.92), -1.07 ± 0.13 (RO: 0.95), -0.85 ± 0.18 (RO: 0.89), and 0.01 ± 0.09 (RO: 0.06), respectively.

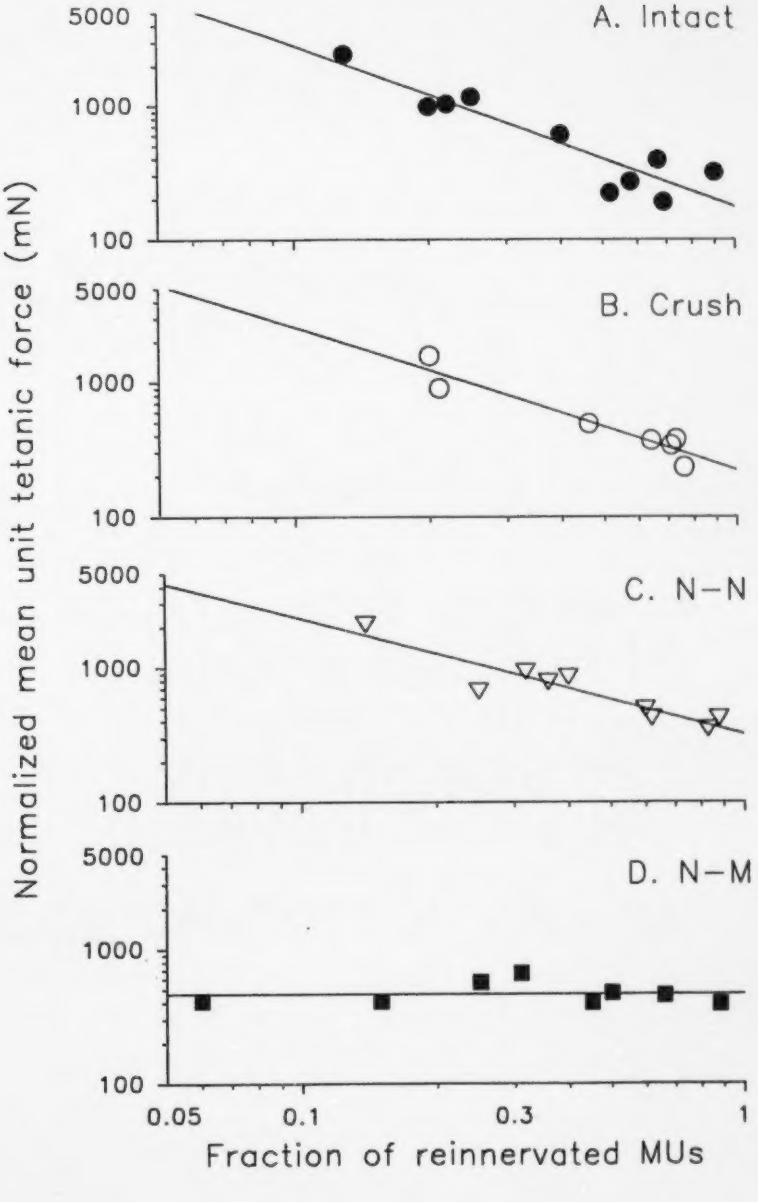
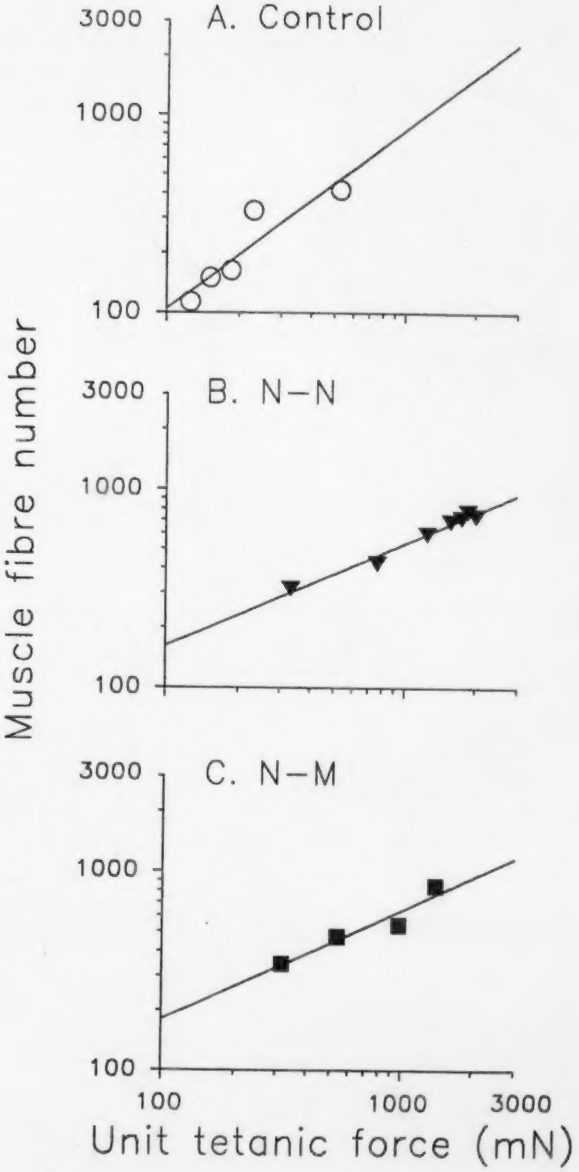

A. Intact
B. Crush
C. N−N
D. N−M
5000
1000
100
Normalized mean unit tetanic force (mN)
0.05
0.1
0.3
1
Fraction of reinnervated MUs

Figure 3.11.

Number of glycogen-depleted muscle unit fibers varies positively with unit tetanic force for 5 normal MUs (A), and 7 MUs in reinnervated muscles after N-N suture (B) and for 4 MUs in reinnervated muscles after N-M suture (C). The strong positive correlation between unit force and muscle fiber number indicates that unit IR is an important determinant in unit tetanic force. All regression lines are significantly different from zero ($p<0.01$).


A. Control
B. N–N
C. N–M
3000
1000
100
Muscle fibre number
100
1000
3000
Unit tetanic force (mN)

(RO: 0.92) and is significantly different from zero. A significant correlation between fiber number and unit tetanic force was also observed in reinnervated muscles after N-N (Fig. 3.11B) and N-M suture (Fig. 3.11C). The slopes (± S.E.) of the regression lines were 0.52 ± 0.04 (RO: 0.99) and 0.55 ± 0.12 (RO: 0.96) for N-N and N-M sutures, respectively, and were not significantly different from each other but were significantly different from zero ($p<0.01$). IR is therefore a significant determinant of unit tetanic force in normal and reinnervated cat MG muscles in agreement with normal cat TA (Bodine et al. 1987), rat TA (Tötösy de Zepetnek et al. 1992a) and rat soleus (Chamberlain and Lewis 1989) as well as reinnervated rat TA muscles (Tötösy de Zepetnek. 1992a).

3.4 DISCUSSION

This study shows that regenerating nerves have the same capacity as normal to form enlarged MUs to compensate for a decreased number of MUs (ie. enlargement of 5-8 times). Only when regenerating nerves do not branch within intramuscular nerve sheaths is the capacity compromised. Thus the capacity for MU enlargement depends on how many regenerating motor axons reinnervate the denervated muscle and growth environment of nerve branching. Although the relative size of S,FR,FF units remains the same after MU enlargement, namely $S \leq FR < FI \leq FF$, the smaller S units increase relatively more than FF units. These results provide important insights into 1) understanding mechanisms governing the success of neuromuscular reinnervation and 2) providing a rationale for the surgical management of peripheral nerve injuries.

3.4.1 Success of muscle reinnervation in experimental models

Recovery of muscle force is generally good after nerve crush (Sunderland 1978) and nerve section after adjoining the proximal and distal stumps (self-reinnervation; Foehring et al., 1986b; Gordon and Stein 1982ab, see also Fig. 3.2). Even after cross-anastomosing stumps of different nerves (cross-reinnervation; Bagust and Lewis 1974; Bagust et al. 1981; Chan et al. 1982; Dum et al. 1985; Foehring et al. 1987a; Gordon et al. 1986b; Lewis et al. 1982) muscles recovered at least 80-100% of the contralateral force.

3.4.2 Resolution of MU size

Experiments in the cat, that examined recovery at the MU level, have shown that the mean unit force in muscles was normal or slightly less than normal even though the number of reinnervated MUs was generally only two-thirds that of normal (Bagust and Lewis 1974; Bagust et al. 1981; Dum et al. 1985: Lewis et al. 1982; Tötösy de Zepetnek et al. 1992). Findings that there were actually fewer large MUs than normal and that unit force is the same or smaller than normal values suggested that motoneurons are intrinsically limited to supply a finite number of muscle fibers (Bagust and Lewis 1974; Chan et al. 1982). However studies of MU enlargement after partial denervation (Chapter 2; Luff et al. 1988; Luff and Torkko, 1990;) suggest that increased unit force may be difficult to detect until a substantial reduction in the MU number occurs. Normally, there is a 100- fold range in unit force and even when a large sample of MUs is taken, a 2-fold increase in unit force may go undetected.

Errors in determining MU number and mean unit force compound to obscure the 2-fold increase because estimates of MU numbers are commonly obtained, as in this study, by dividing muscle force by mean unit force. Thus mean unit force is used to determine both size and number of MUs. Accuracy in determining mean unit force depends on adequate sampling. Samples of between 30 to 40 MUs are unusual and even such large samples represents only 10 to 20% of the MG unit population (Boyd and Davey, 1968). More commonly, samples from different muscles are pooled together and as a result a 2- fold difference in unit force may be obscured. Secondly, unit force represents the number of muscle fibers reinnervated, or IR, only if reinnervated muscle fibers recover their original size (see below).

Our findings that mean unit force only increased significantly in muscles after N-N suture if the number of reinnervated MUs is reduced by more than 50% of normal are consistent with this reasoning. Therefore earlier findings that unit force did not increase significantly in reinnervated muscles in the earlier studies may be attributed to poor resolution of the 2-fold increase in MU size. A 2-fold increase is expected if more than 50% of the MUs reinnervate the muscle. Only under experimental conditions in which we reduced MU numbers to less than 50% has the capacity of intact and regenerating nerves to enlarge MUs been resolved.

Regenerating nerves after crush injuries have the same capacity to increase MU size as intact nerves in partially denervated muscles such that the enlarged MU size was sufficient to reinnervate all denervated muscles so long as 15% of the normal complement of MUs reinnervate the muscle. Thus, cutting nerves does not change the capacity of the motoneuron to branch. The finding that enlargement of unit force and recovery of whole muscle force and weight was less when the endoneurial tubes were disrupted and nerves regenerated after N-N repair suggests that the severity of the nerve injury compromises the ability of regenerating nerves to reinnervate all available denervated muscle fibers when the MU number is reduced (Figs. 3.2 and 3.4). However, unit force is a reasonable measure of number of muscle fibers reinnervated only if muscle fibers recover their former size. After nerve section, in contrast to nerve crush injuries, mean reinnervated muscle fiber size was lower than normal. Reduced muscle recovery after N-N suture could then be attributed to reduced reinnervated muscle fiber size and not to a compromised ability of cut nerves to enlarge their MUs by increasing the number of muscle fibers reinnervated. These findings therefore provide strong evidence that regenerating nerves have the same capacity as intact nerves to enlarge their MU size by increasing IR, irrespective of the type of nerve injury or whether or not the regenerating nerves follow their original endoneurial sheaths. The finding that MU enlargement after N-M suture was significantly curtailed and regenerating motor axons formed smaller MUs indicates that the branching capacity of regenerating nerves is more limited when they are force to grow along muscle fibers (Fig. 3.4). Thus, it is the growth environment, and not the status of the nerve, that determines the capacity for MUs to enlarge.

3.4.3 Factors controlling IR

There are two possible ways, that are not mutually exclusive, for MUs to increase their IRs, namely: 1) supply more muscle fibers within the unit territory, which would increase the muscle unit fiber density and/or 2) increase the size of the unit territory. As described by Kugelberg et al. (1970), an increased muscle unit density may reflect an increased number of terminal branches close to the muscle fibers while a more extensive territory would be the result of more distant axonal branching in the larger intramuscular nerve trunks (Kugelberg et al. 1970; Gordon et al. 1991). The limit of a 5-8 fold increase in MU size by sprouting in partially denervated muscles has been attributed to an inability of MUs to increase their territories by branching from large proximal trunks while the terminal branches are limited to innervate muscle fibers only within the unit territory (Kugelberg et al. 1970; Gordon et al. 1991). This same principle could explain why reinnervated MUs are more limited in their capacity to enlarge after the nerve is completely severed and sutured directly to the muscle fascia (N-M suture; Fig. 3.3D). After N-M suture, the reinnervated MU territories tend to be smaller than normal, possibly because of less extensive intramuscular branching along the proximal portion of the distal nerve trunk (see Chapter 4 for details). Due to the extensive terminal branching muscle unit fibers become progressively more clumped within the small MU territory as the number of MUs decline. When less than 20% of the MUs reinnervate the denervated muscle, there is an insufficient number of muscle fibers within the smaller unit territory for the MU to enlarge in size to the same extent as intact and regenerated axons following nerve crush. As a result, when the number of MUs is reduced by more than 50% whole muscle force fails to fully recover (Fig. 3.2A, see also Gordon and Stein, 1982ab). The failure of regenerating nerves to enlarge MU size when the axons do not follow intramuscular nerve sheaths shows that the sheath is essential for axon branching. It is likely that the nerves enter the sheaths at some point since the regenerating axons find their denervated endplates which are the preferred sites of reinnervation (Fu and Gordon, unpublished observations).

If CSA and SF are not greatly changed by the experimental procedure unit force is a reasonable measure of IR (Tötösy de Zepetnek et al. 1992a; Gordon et al 1991). The parallel shift in the unit force distribution (on logarithmic scales) to larger forces in partially denervated muscles (Fig. 3.3A) shows that all MUs increased in size by the same factor and muscle fibers recover their former size (Fig. 2.8). These results show the largest diameter motor axons sprout more to supply a larger number of muscle fibers as compared to smaller axons to preserve the normal size dependent branching (Chapter 2). Following nerve crush or nerve section the shift was not parallel. The small MUs appeared to increase in size more than the larger MUs (Fig. 3.3B,C). Is this differential increase of S units due to an increased capacity to increase IR or to differences in recovery of muscle fiber size and/or SF?

The tetanic force of the S units increased more, and the FF units less, than expected in muscles where the nerve was either crushed (Fig. 3.5B) or cut and sutured to its distal stump (N-N suture; Fig. 3.5C) or directly to the muscle fascia (N-M suture; Fig. 3.5D). These observations are consistent with previous findings that force of small MUs increased more than larger MUs in reinnervated muscles (Bagust and Lewis, 1974; Desypris and Parry, 1990; Foehring et al. 1986a; Gordon and Stein, 1982b; Lewis et al. 1982; Tötösy de Zepetnek et al. 1992a). Our direct measures of IR, by counting glycogen-depleted muscle unit fibers, confirms that force correlates well with IR in reinnervated muscles (Fig. 3.11) showing that IR remains an important determinant of unit force. However, because FOG and FG fibers were significantly smaller than normal (Fig. 3.7, see also Foehring et al. 1986a) enlargement of MU size by an increased IR in F units (FR and FF) is underestimated by force measurements. Reduced FOG and FG fiber CSA accounts for some, but not all of the considerably lower than expected increase in unit force of reinnervated F units, particularly FF units (Fig. 3.5). The smaller enlargement of F units compared to S units is therefore also due to the lesser ability of F units to increase their IR (see also Foehring et al. 1986a) if SF is transformed by the reinnervating nerve.

It is possible that the restricted reinnervated unit territory may account for the differential capacity for S units to enlarge more than F units since a reduced territory size would decrease the availability of muscle fibers for reinnervation. This decreased availability would effect larger motoneurons to a greater extent compared to smaller axons. For example, if all reinnervated MUs increased in size by 4 times, which would be required if only 25% of the MUs reinnervate the muscle, then an axon which originally supplied 400 muscle fibers would have to innervate 1200 fibers for an increase of 800 fibers. A smaller motoneuron which originally innervated 40 fibers would only have to innervate 80 fibers to increase its normal innervation to 120 (4 times that of normal). If the larger motoneurons did not have access to 800 denervated fibers then it is conceivable that the smaller axons increased their innervation by capturing a greater number of fibers than would be expected.

3.4.4 Factors controlling reinnervated muscle fiber size

Muscle fiber CSA in reinnervated muscles recovers to normal after crush injury (ie. $SO \leq FOG < FG$), but not following nerve section and self-reinnervation after N-N and N-M suture (Fig. 3.7; see also Foehring et al. 1986a). This incomplete recovery of FOG and FG muscle fiber CSA contrasts with the complete recovery in the same muscle after cross-reinnervation with the lateral gastrocnemius-soleus (LGS) nerve (Foehring et al. 1987). After cross-reinnervation of MG by flexor motoneurons the mean CSA of all fiber types was the same because SO and FOG fibers increased significantly in size (Gordon et al. 1988). These differences may be reconciled by different mechanical loading of the reinnervated muscle fibers under the different reinnervation conditions rather than differences in neural control of muscle fiber size. Mechanical loading is known to increase fiber size particular if the muscle contracts isometrically (Edgerton 1978). Increased load is associated with fiber hypertrophy while muscle contraction in a shortened position leads to reduced muscle fiber CSA and reduction in force. The apparent hypertrophy of the smaller SO and FOG fibers in the study of Gordon et al (1988) may be related to the abnormally high degree of co-contraction of the extensor and flexors hindlimb muscles in these cats during walking (Gordon et al. 1986b) which would impose significantly more resistance to movement and thereby increasing the load of the muscle fibers. After self-reinnervation of MG muscle conduction delays imposed by lower than normal conduction velocities below the suture line (Horch and Lisney 1981; Gordon and Stein 1982ab) may lead to asynchrony of contraction of the MG muscle with its synergists. As a result, the MG will contract significantly latter than its synergists and therefore the muscle fibers will contract in an all ready shortened position and against a smaller load. This appears to be most detrimental for the FOG and FG fibers which decrease in size while SO fibers recover to normal (Fig. 3.7). Conduction velocity distal to the site of a nerve crush injury recovers to near normal values (Cragg and Thomas 1964; Horch and Lisney 1981). As a result, the relative timing of the contraction of the MG muscle with its synergists is normal, and consequently, the larger muscle fiber do not atrophy (Fig. 3.7). Following cross-reinnervation of the MG and LGS muscles (study of Foehring et al. 1986ab) the conduction velocity of all 3 muscles will be decreased by a similar extent, whereby the synchrony of muscle fiber contraction remains normal and muscle fiber size recovers more completely.

From a clinical standpoint, these results show that at least 35% of the normal complement of MUs are required to ensure reinnervation of all denervated muscles. Surgery is often undertaken to increase the number of regenerating motor nerves which at the same time can decrease the accuracy of reinnervation. Since function may be more detrimentally affected by misdirection of nerves than muscle weakness, our findings suggest that surgical interventions, such as grafting, should be avoided if alternative strategies allow for reinnervation of a minimum of 35% of the normal complement of MUs. Consideration of the altered mechanical loading of reinnervated muscle fibers is important in rehabilitation of patient who suffer from peripheral nerve injuries. Thus, the capacity for regenerating nerves to expand their MU size and mechanical properties are two very important aspects which must be considered in the surgical and rehabilitative management of nerve injuries.

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4. SPATIAL DISTRIBUTION OF MOTOR UNIT FIBERS IN NORMAL AND REINNERVATED CAT MG MUSCLE

4.1 INTRODUCTION

Typically, two to three fiber types, Type I, IIa and IIb, can be demonstrated in skeletal muscle using either myosin ATPase (mATPase) (Brooke and Kaiser 1970, Guth and Samaha 1970) or a combination of mATPase and metabolic enzymes (Peter et al. 1972). Normally the types are randomly distributed in a characteristic mosaic pattern. This mosaic pattern is widely seen in many different muscles in several animal species. In man, the mosaic distribution is generally used as the normal standard of comparison in diagnosing neuromuscular pathology. Tendency of the same fiber types to cluster into groups (fiber type-grouping or clumping) is a general finding in muscle biopsies for a wide group of neuromuscular disorders where muscle denervation and reinnervation is suggested. These include muscular dystrophies, poliomyelitis, Bell's palsy, severe neuropathies and of course peripheral nerve injuries where muscles may be completely or partially denervated (Dubowitz and Brooke, 1973; Sunderland, 1978).

Muscle fiber type-grouping is generally found in reinnervated muscles in experimental animal models of peripheral nerve section and repair (Dubowitz 1967; Gordon et al. 1988; Karparti and Engel, 1968; Kugelberg et al. 1973). It has been widely assumed that fiber type-grouping represents clustering of fibers belonging to a single motor unit (MU). For example, clumped fibers of the same type were analyzed to conclude that reinnervated MUs were more similar in metabolic enzyme profiles than non-unit fibers and used as evidence for neural control of muscle fiber phenotype (Nemeth et al. 1986). This assumption is not necessarily valid. Muscle fibers belonging to a single MU (therefore supplied by one motoneuron) can be identified by depleting them of their glycogen stores and visualizing them histochemically as negatively stained fibers using the Periodic acid Schiff reaction (Edstrom and Kugelberg 1968). Having developed this technique, Kugelberg et al. (1970) showed that reinnervated muscle unit fibers were indeed clumped together. However, examination of all muscle unit fibers showed that they are distributed in several clumps within a defined territory (Tötösy de Zepetnek et al. 1988; Tötösy de Zepetnek et al. 1990). As a result, only some, and not all, adjacent fibers in a clump that are of the same histological type actually belong to a single MU.

While fiber type clumping is striking in the reinnervated rat muscles that have been studied; tibialis anterior (TA; Kugelberg et al. 1970; Fu et al. 1992; Parry and Wilkinson 1990; Tötösy de Zepetnek et al. 1992;), extensor digitorum longus (EDL; Albani et al. 1988), lateral gastrocnemius (LG) and soleus (Gillespie et al. 1987), it is less striking, although evident, in the larger cat hindlimb muscles (Dum et al. 1985a: Foehring et al. 1986; Gauthier and Dum 1973; Gordon et al. 1986). In fact, we were struck by the absence of clumping in the reinnervated MG (Gordon et al. 1988). Spatial analysis of fiber type-grouping in self-reinnervated cat medial gastrocnemius (MG) muscle in a recent study found that grouping is barely significant using the Monte Carlo simulation technique to analyze spatial distribution (Nemeth et al. 1992).

How can the data from the smaller rat and larger cat muscles be reconciled and how well do the experimental models represent the human muscle? Are the differences simply due to species difference or does the extent of type-grouping reflect underlying differences in the pattern of branching of the regenerating motoneurons? We developed an idea, originating from the work of Kugelberg et al. (1970), that the smaller territory that the reinnervated MU occupies in the rat muscles reflects a change in the normal pattern of branching of regenerating nerves (Gordon et al. 1991). The more restricted territory was suggested to arise because the first order branching of the nerves within the intramuscular sheaths (proximal branching) was less than normal and that more terminal branching (distal branching) predominates during muscle reinnervation. This same hypothesis may explain why there is less clumping in larger muscles such as those in the cat hindlimb. Terminal branching would presumably be restricted by epimysial barriers so that MU enlargement might be expected to occur within a restricted territory. Where MU territories are large, perhaps fiber clumping within the territory would not be obvious unless MUs were significantly enlarged under conditions where fewer than normal nerves make functional connections.

To test this idea we have examined the size of reinnervated unit territories and the degree of clumping of muscle unit fibers within the territory under conditions where the number of MUs was experimentally reduced. We cut and resutured the nerve of the cat MG muscle at the same time as reducing the number of available regenerating nerves by cutting one of the two contributing ventral roots that supply motor axons to the MG. Our expectation is that the extent of fiber type-grouping should parallel changes within single MUs such that as the fibers within a single MU become more clumped, type-grouping will be more evident. We also examined the spatial distribution of muscle unit fibers under conditions where we have previously shown that MU enlargement is severely restricted (ie. nerve-muscle suture; Chapter 3) where we predict that MU territories will be smaller and muscle unit fibers will be significantly more clumped.

MG muscles were reinnervated after cutting the MG nerve and either suturing its proximal stump to the distal stump (N-N suture) or directly to the muscle fascia (N-M suture). The number of regenerating nerves was also experimentally reduced by sectioning 1 of the 2 spinal roots contributing motor axons to the MG muscle. As a result, the number of reinnervated MUs in the MG muscle (% MUs) varied considerably between animals ranging from 2 to 88% that of normal. In Chapter 3 we showed that mean unit force increased inversely with the number of reinnervated MUs, however the increase was substantially higher in muscles after N-N as compared to N-M sutures. By analyzing the territories and distribution of muscle unit fibers in reinnervated muscles after N-N or N-M suture it may be possible to determine what factors fostered the greater enlargement capacity of MUs in muscles with N-N suture. Some of these results have appeared previously in abstract form (Rafuse et al. 1989).

4.2 METHODS

A total of 19 cats were used in this study. This number includes: 5 normal and 14 experimental cats in which the MG muscle was denervated by transection of the MG nerve. The cut nerve was either sutured to its distal stump (N-N suture, n=9) or directly to the muscle fascia (N-M suture, n=5). All cats were similar in weight (range: 2.6-3.6 kg) with a mean (± S.E.) of 3.2 ± 0.11 kg. Initial surgery, preparation for acute experiment, characterization of MUs, glycogen-depletion protocol, histochemical analysis, and muscle fiber CSA measurements are described in detail in Chapter 3.

4.2.1 Spatial analyses of glycogen-depleted muscle fibers

The position of the glycogen-depleted muscle fibers was located on serial cross-sections, 10 μm thick, taken from each of the five blocks cut along the muscles's longitudinal axis (L1-L5, Fig. 4.1A). Glycogen-depleted muscle fibers were identified in muscle cross-sections as negatively stained fibers after staining with the periodic acid Schiff reaction (Pearse 1961). The size of the unit territory within the longitudinal axis was estimated by recording the presence or absence of glycogen-depleted fibers in each block. Camera lucida drawings of the glycogen-depleted muscle fibers were made by projecting the original PAS stained slide onto a white screen with the use of a microprojector. From the cross-section containing the largest number of depleted muscle fibers the transverse territory of the MU (ie. the region of the whole muscle cross-section containing all fibers belonging to the isolated MU) was determined by connecting

Figure 4.1.

Schematic representation of cat MG muscle as viewed from the plan and longitudinal axes. The MG muscle was cut into 5 blocks along its longitudinal axis (L1 to L5) to be later sectioned transversely (B) to identify the position of glycogen-depleted MUs along this axis. The transverse cross-section containing the greatest number of depleted muscle fibers was used for determining the relative number of muscle unit fibers (Innervation ratio: IR) and to analyze the unit territory and spatial distribution of depleted fibers. The size of the unit territory was determined by connecting the outlying fibers with straight lines to form the smallest convex area that contains all the depleted fibers (B).

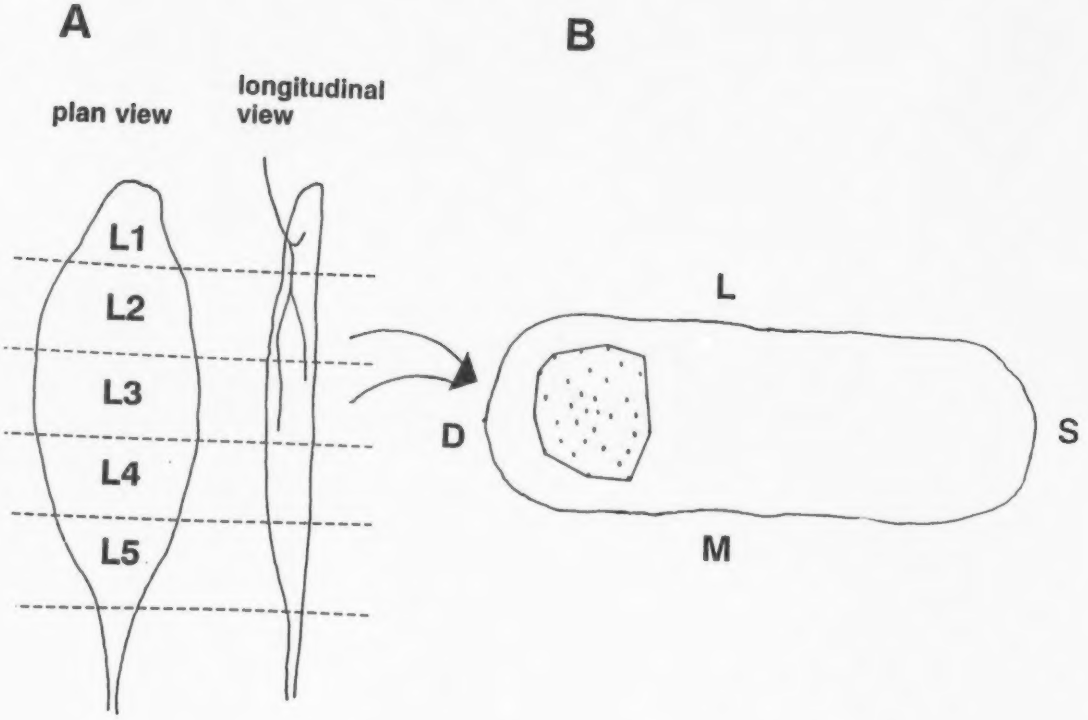
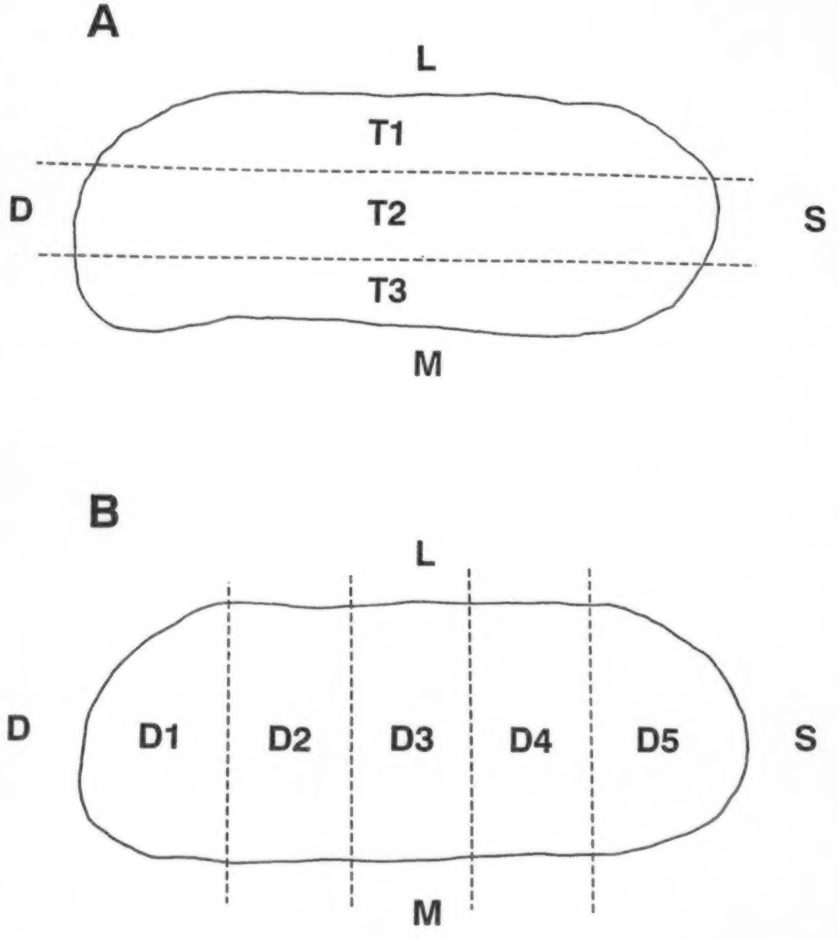

A
B
plan view
longitudinal view
L1
L2
L3
L4
L5
L
D
S
M

Figure 4.2.

Transverse cross-sections of the whole muscle were divided into 3 regions (T1 - T3) extending from the lateral to medial surfaces of the muscle (A) and into 5 regions (D1 - D5) extending from deep to superficial to quantify the location of the depleted MUs within these axes. L, lateral; M, medial; D, deep; S, superficial.


A
L
T1
D
T2
S
T3
M
B
L
D
D1
D2
D3
D4
D5
S
M

the outlying fibers with straight lines to form the smallest convex area that contains all the muscle unit fibers (for example see Fig. 4.1B). The muscle cross-section was divided into three regions, from the muscle's medial to lateral surfaces (T1-T3, Fig. 4.2A), and into five regions extending from the deep to superficial regions (D1-D5, Fig. 4.2B), to assess the location of the unit territory within the transverse plane.

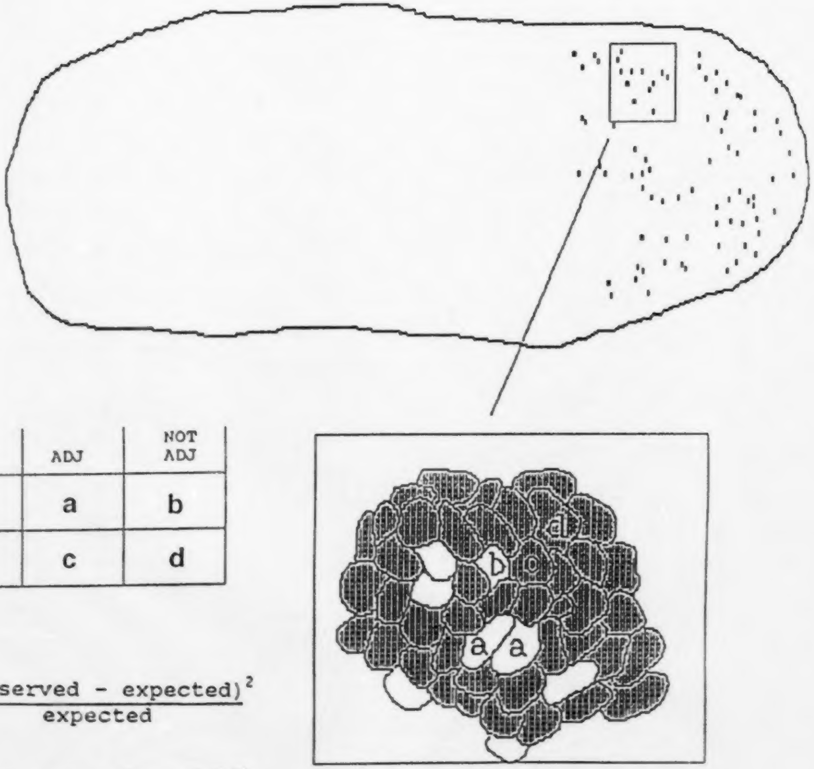
The degree of muscle unit fiber clumping within the unit territory (ie. the extent to which glycogen-depleted muscles fibers are adjacent to each other) was determined by calculating: 1) fiber density, 2) number of adjacencies between unit fibers, and 3) Chi-square value to test for randomness between glycogen-depleted and non-depleted muscle fibers.

The degree of clumping between unit fibers was determined by counting each glycogen-depleted fiber and recording whether or not it was adjacent to another depleted fiber. Two fibers were considered to be adjacent when their fiber boundaries were in contact. If the depleted fiber was adjacent to another depleted fiber the total number of fibers within that group was counted. In this manner all muscle unit fibers were counted once and the number of fibers that occurred as "singles" (not adjacent to another depleted fiber) or were in groups of two, three, four, etc was recorded. A cumulative frequency distribution of the number of adjacencies was plotted to determine how many muscle fibers are grouped together when 80% of the population is counted (adj. @ 80%). The maximum number of depleted fibers within a single group was also determined.

Chi-square analysis was performed on the distribution of glycogen-depleted fibers

Figure 4.3.

Classification of depleted and non-depleted muscle fibers used in Chi-square analysis. Fibers were classified into 4 categories according to 2 properties. 1) belonging to the depleted MU (open fibers; a,b) or not belonging to the depleted MU (shaded fibers; c,d) and 2) being adjacent to a depleted fiber (a,c) or not adjacent to a depleted fiber (b,d). Chi-square analysis was performed by setting up a 2 X 2 contingency table to determine the expected frequency of fibers in each category.



	ADJ	NOT ADJ
DEPL	a	b
NOT DEPL	c	d

$$\chi^2 = \Sigma \frac{(\text{observed} - \text{expected})^2}{\text{expected}}$$

Significant if $X^2 > 3.84$ $(P = 0.05)$

to determine whether the observed number of adjacencies between glycogen-depleted muscle fibers was significantly greater than would be expected if the fibers were randomly distributed (see Bodine et al. 1988). To perform the analysis, 2 to 6 photomicrographs were taken of regions with the highest density of depleted fibers within the unit territories. In this manner between 16% to 100% (X: 42 ± 6.0%) of the total number of glycogen-depleted muscle fibers in the whole muscle cross-section were photographed. All fibers in the photomicrographs were included in the Chi-square analysis. As described in detail by Bodine et al. (1988), Chi-square analysis is performed by setting up a 2 X 2 contingency table to test whether each fiber is first a depleted or non-depleted fiber and second whether the fiber is adjacent or not adjacent to another depleted fiber (Fig. 4.3). In so doing, each "observed" fiber can be classified into 1 of 4 categories. This is shown diagrammatically in Fig. 4.3. The expected frequency for each category is calculated by multiplying the two values in a single row or column and then dividing this sum by the total number of observations (ie. muscle fibers). For example, the expected frequency in category a (Fig. 4.3) is:

$$(a + c) \times (a + b) / (a + b + c + d)$$

The observed values in each category were then compared to the expected values for that category, and a Chi-square statistic was calculated according to

$$(\text{observed} - \text{expected})^2 / \text{expected}$$

A Chi-square value larger than 3.84 ($p<0.05$) suggests that there is a greater number of adjacencies between glycogen-depleted fibers than would be expected if they were randomly distributed. Chi-square values were calculated for three normal and 11 reinnervated MUs (Table 4.3). A Chi-square value could not be determined for one control MU (Control #1; Table 4.3) since all fibers occurred as "singles" (category b; Fig. 4.3). Similarly, a Chi-square value could not be determined for one reinnervated MU (N-N 9; Table 4.3) because all depleted fibers were adjacent to each other (category a; Fig. 4.3).

4.2.2 Muscle fiber histochemistry, fiber CSA measurements and regionalization of muscle fiber types.

Cross-sections through the mid-belly of the MG muscle were stained for mATPase activity with acid or alkaline preincubation as outlined in detail by Gordon et al. (1988). Muscle fiber types were identified as slow-oxidative (SO), fast-oxidative glycolytic (FOG), and fast-glycolytic (FG) according to the criteria of Brooke and Kaiser (1970) although the nomenclature of Peter et al (1972) is used throughout the paper. Muscle fiber CSAs were measured with the use of a microcomputer digitizing software program (JAVA, Jandel Scientific). This system was linked from the computer to a microscope via a video camera. The CSA of between 159 to 198 of the glycogen-depleted muscle fibers and between 290 to 1703 non-depleted fibers were measured. CSAs of the whole muscle cross-section and unit territories were measured by planimetry on a digitizing tablet (Summagraphics MM1200 series).

The total number of muscle fibers within a single muscle cross-section was estimated by dividing the total muscle CSA by the mean CSA of all muscle fibers measured in the cross-section. The total number of muscle fibers was used to estimate the mean number of muscle fibers innervated by each motoneuron (innervation ratio; IR) in normal and reinnervated muscles. The estimated IR was calculated by dividing the number of muscle fibers by the number of MUs in the muscle. Normal MG muscles were considered to contain 280 MUs (Boyd and Davey 1968). The number of MUs in reinnervated muscles was calculated by dividing whole muscle tetanic force by the mean tetanic force of the MUs sampled in the muscle (see Methods in Chapter 3 for details).

To quantify the regionalization of SO fibers in both normal and reinnervated muscles, the whole muscle cross-section was divided into 5 sections from the deep to the superficial regions of the muscle (Fig. 4.2B). A circular area (3.64 mm^2) was drawn in the center of each of the 5 sections. The total number of SO fibers counted in each circular area was determined and comparisons between each section was used to quantify regionalization of SO fibers across the deep to superficial regions of the MG muscle cross-section. As calculated for the glycogen-depleted muscle fibers, the adjacencies of SO fibers within each circular area was determined in order to quantify the extent of SO fiber type-grouping. Adjacencies extending beyond the circular area were not included in the counts.

Table 4.1. Summary of glycogen-depleted MUs. Size and distribution.

Condition	Unit Type	% MUs	Po (mN)	Unit Fiber Number	Depleted Mean Fiber CSA (μm^2)	Long. area	Traverse Terr.	D-S Terr.
Control 1	S	100	151	150	1261 ± 30	L1	T2,T3	D1
Control 2	S	100	185	163	2025 ± 40	L1,L2,L3,L4	T1,T2,T3	TD1,D2,D3
Control 3	FF	100	229	325	5709 ± 92	-	T1,T2,T3	D1,D2,D3
Control 4	FF	100	125	134	5556 ± 92	L1,L2,L3	T1,T2,T3	D1,D2
Control 5	FF	100	528	416	6538 ± 80	L1,L2,L3	T1,T2,T3	D3,D4,D5
N-N 1	FI	88	333	329	4752 ± 111	L2,L3,L4,L5	T1,T2	D1,D2,D3
N-N 2	FR	62	364	219	4161 ± 220	L2,L3,L4	T1,T2	D4,D5
N-N 3	FI	60	766	485	4799 ± 106	-	T1,T2,T3	D3,D4,D5
N-N 4	FI	40	1757	776	5436 ± 140	L2,L3,L4	T1,T2,T3	D2,D3
N-N 5	FR	36	1576	690	3499 ± 84	L2,L3	T2,T3	D1,D2
N-N 6	FF	32	1867	844	4326 ± 85	L2,L3,L4,L5	T1,T2,T3	D1,D2,D3,D4
N-N 7	FF	25	2019	789	4421 ± 206	L1,L2,L3	T1,T2,T3	D3,D4,D5
N-N 8	FI	14	1254	819	2719 ±81	L2,L3,L4	T1,T2,T3	D1-D5
N-N 9	FF	2	5320	2025	6246 ± 147	L3,L4	T1,T2,T3	D1,D2,D3
N-M 1	FI	66	391	495	2838 ± 82	L1,L2,L3	T2,T3,	D2,D3,D4
N-M 2	FF	50	973	601	4646 ± 102	L1,L2,L3,L4	T1,T2,T3	D1,D2
N-M 3	FI	40	536	457	2892 ± 71	L2,L3,L4	T1,T2,T3	D1,D2,D3
N-N 4	FR	45	316	587	1233 ± 37	-	T2,T3	D2,D3,D4,D5
N-M 5	FI	6	1800	1449	6310 ± 118	-	T2,T3	D1,D2,D3

Mean values ± S.E. N-N, nerve-nerve suture; N-M, nerve-muscle suture,; % MU, number of innervating MUs (% of normal); Po, unit tetanic force; Depleted Mean Fiber CSA, cross-sectional area of muscle unit fibers; Long. Terr., unit longitudinal location; Traverse Terr. unit location on traverse plane; D-S Terr, unit location along deep to superficial axis.

Table 4.2. Glycogen-depleted MU and territory size.

Condition	% MUs	Muscle CSA	Terr. CSA (abs. mm^2)	Terr. CSA (%)	Mean Muscle Fiber CSA (μm^2)	Total Muscle Fiber Number	Estimated Mean IR
Control 1	100	203	14	7	3736 ± 106	54336	194
Control 2	100	224	52	23	3801 ± 90	58931	210
Control 3	100	250	45	25	3703 ± 90	67513	241
Control 4	100	276	33	12	4503 ± 104	61292	219
Control 5	100	270	70	32	4570 ± 85	59080	211
N-N 1	88	192	30	16	2663 ± 24	72099	293
N-N 2	62	195	17	11	3471 ± 70	59061	340
N-N 3	60	203	44	22	3002 ± 52	67622	403
N-N 4	40	213	54	25	2666 ± 49	79895	713
N-N 5	36	180	25	19	2885 ± 69	62392	619
N-N 6	32	208	96	46	3070 ± 47	67752	756
N-N 7	25	230	49	54	3520 ± 66	65341	933
N-N 8	14	90	63	43	1687 ± 25	53349	2238
N-N 9	2	16	16	100	5972 ± 90	8707	1555
N-M 1	66	170	22	20	2714 ± 60	62638	339
N-M 2	50	161	35	22	3158 ± 28	50982	364
N-M 3	31	136	29	22	2487 ± 44	54684	616
N-M 4	25	68	14	16	1577 ± 41	43120	428
N-M 5	6	66	24	36	6215 ± 95	10619	632

Mean values ± S.E. N-N, nerve-nerve suture; N-M, nerve-muscle suture, % MU, number of innervating MUs (% of normal); Muscle CSA, whole muscle cross-sectional area, Terr. CSA (abs.), absolute area of unit territory; Terr. CSA (%), unit territory size as a % of the totoal muscle CSA; Estimated Mean IR, estimated mean innervation ratio.

Table 4.3. Summary of density and clumping of muscle unit fibers

Condition	% MUs	Terr. CSA (abs. mm^2)	Unit Fiber Number	Density (fibers/ mm^2)	Unit Fiber Adj. @ 80%	Max. Number Adj.	Chi-square value
Control 1	100	14	150	11	1	1	NA
Control 2	100	52	163	3.1	1	5	3.8
Control 3	100	45	291	6.5	1	3	
Control 4	100	33	179	5.4	1	3	1.6
Control 5	100	70	416	5.9	1	3	4.1
N-N 1	88	30	329	11	6	17	22
N-N 2	62	17	219	13	5	21	12
N-N 3	60	44	485	14	12	37	33
N-N 4	40	54	776	14	12	27	33
N-N 5	36	25	690	28	9	50	-
N-N 6	32	96	844	9	4	17	82
N-N 7	25	49	789	16	4	16	86
N-N 8	14	63	819	13	4	15	48
N-N 9	2	16	2025	126	NA	NA	NA
N-M 1	66	22	495	23	8	60	94
N-M 2	50	35	601	17	40	158	205
N-M 3	31	29	457	16	20	84	176
N-N 4	25	14	587	42	12	20	
N-M 5	6	24	1449	60	NA	NA	528

N-N, nerve-nerve suture; N-M nerve-muscle suture; % MUs, number of innervating MUs (% of normal); Density (fiber/mm^2), ratio of unit fiber number and absolute unit territory cross-sectional area; Unit Fiber Adj. @ 80%, number of adjacencies observed between 80% of the depleted muscle fibers; Max. Number of Adj. Maximum number of adjacencies of depleted fibers within a single group.

4.3 RESULTS

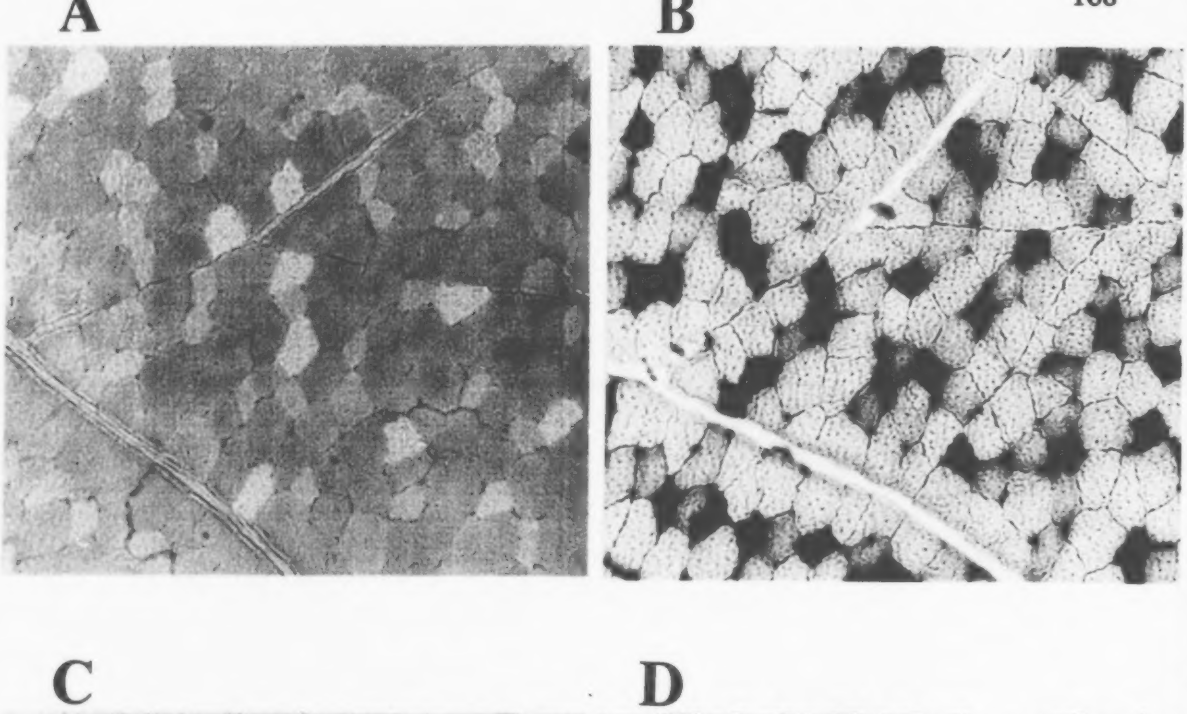
4.3.1 Normal MG nerve branching and muscle compartments

The MG nerve branches into two primary intramuscular trunks along the longitudinal axis of the muscle (Weeks and English 1987) to supply muscle fibers in 1) only the proximal region of the muscle (L1; Fig. 4.1A), and 2) the distal regions of the muscle (L2,L3,L4,L5). Muscle units appear to be localized within these general territories along the longitudinal axis as evident by the localized region of contraction of single MUs and by regionalized distribution of glycogen-depleted muscle unit fibers. For example, stimulation of a small S unit (Control #1; Table 4.1) contracted muscle fibers only in L1 and all of its muscle fibers were histologically identified exclusively in the L1 muscle block. The muscle unit fibers from this S unit are therefore presumably supplied by a motor axon which branched to the deep proximal region of the muscle (L1). The majority of normal MUs occupied a larger region along the longitudinal axis of the muscle (Table 4.1). MUs in Controls #4 and #5 extended through the proximal third while MUs in Control #2 primarily extended through the distal third (L2,L3,L4) of the MG muscle although a few fibers were located in the proximal L1 region.

Within the longitudinal axis of the unit territory one muscle block (eg. L2 for Control #4; Table 4.1) always contained the largest number of glycogen-depleted muscle fibers (see also Burke and Tsairis 1973). The different number and distribution of muscle unit fibers along the longitudinal axis are shown for 1 normal MU (in Control

Figure 4.4.

Photographs of PAS-stained cross-sections showing the muscle fiber distribution of a single normal glycogen-depleted MU in the same region of 2 different blocks (A, L2; C, L3) cut along the longitudinal axis of the muscle. B,D are serial cross-sections stained for myosin ATPase with acid preincubation. The number and distribution of muscle unit fibers differ in the 2 cross-sections. Both photographs (A,C) are taken from the same region in the transverse axis of the muscle.


A
B
C
D
0.1 mm

#5; Table 4.1) in 2 muscle cross-sections (L1 and L2) stained with PAS in Fig. 4.4. Both photographs are taken from the same transverse region of each block.

4.3.2 Normal MU territories

Muscle unit fibers were not distributed across the entire transverse axis of the muscle but rather were located within discrete regions or territories ranging from 14 to 70 mm^2 or between 7 to 32% of the total muscle cross-section (Fig. 4.5, Table 4.2). Normal MUs appeared to occupy territories either in the deep (D1-D3) or superficial regions (D3-D5) of the muscle (Fig. 4.5, Table 4.1). In contrast, 4 of the 5 normal MUs spanned across the entire transverse axis of the muscle from the ventral-lateral surfaces (Fig. 4.5) to occupy regions T1-T3 (Fig. 4.2A; Table 4.1).

The size of the unit territory in normal muscles increased with the number of depleted muscle unit fibers in the cross-section (Fig. 4.6A, Table 4.2). The density (fibers/mm^2) of muscle fibers within the territory was inversely related to the size of the territory (Fig. 4.6D) indicating that larger MUs occupy a proportionately larger area of the muscle compared to smaller MUs.

Normal muscle unit fibers were scattered in the unit territories as previously noted in the cat MG by Burke and Tsairis (1973) and in the rat TA muscle (Kugelberg et al. 1970; Tötösy de Zepetnek et al. 1992). However, fibers are not necessarily evenly distributed throughout the territory and regions of high and low densities can be seen (Fig. 4.5D; see also Bodine-Fowler et al. 1990).

Most normal muscle unit fibers are not adjacent to each other, but rather are

Figure 4.5.

Camera lucida drawings showing the location of the MU territory and distribution of depleted muscle unit fibers within the territory for 3 normal cat MG units. The magnitude of unit force and IR increase from left to right. The drawings are oriented such that the top margin is lateral, the lower is medial and the left and right are deep and superficial, respectively.

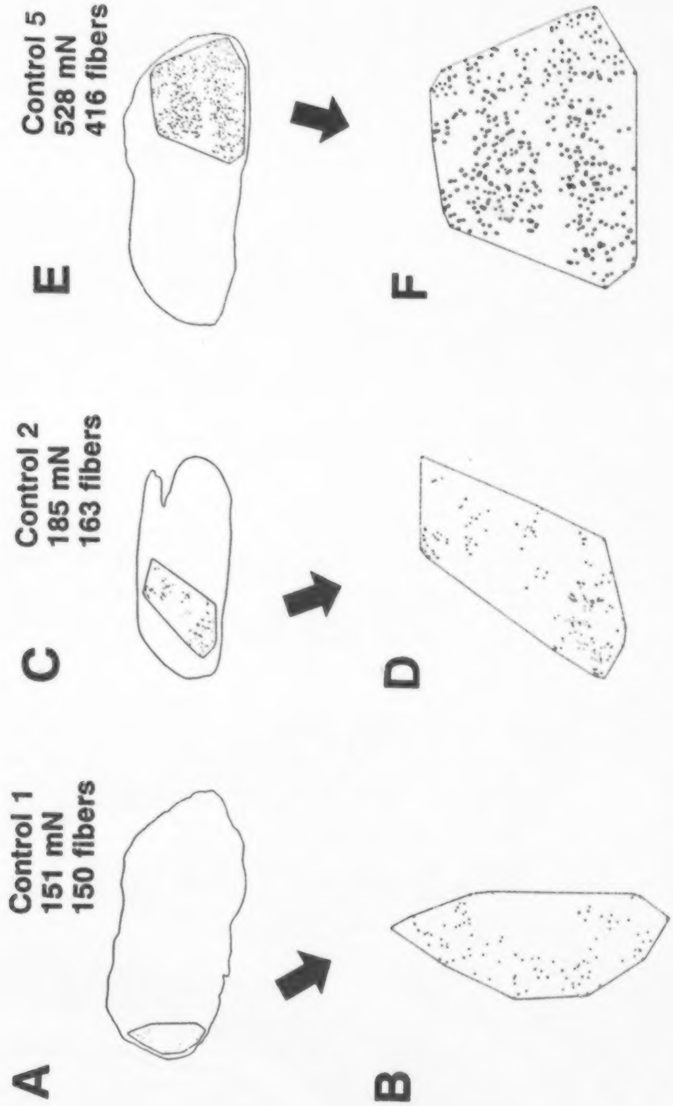
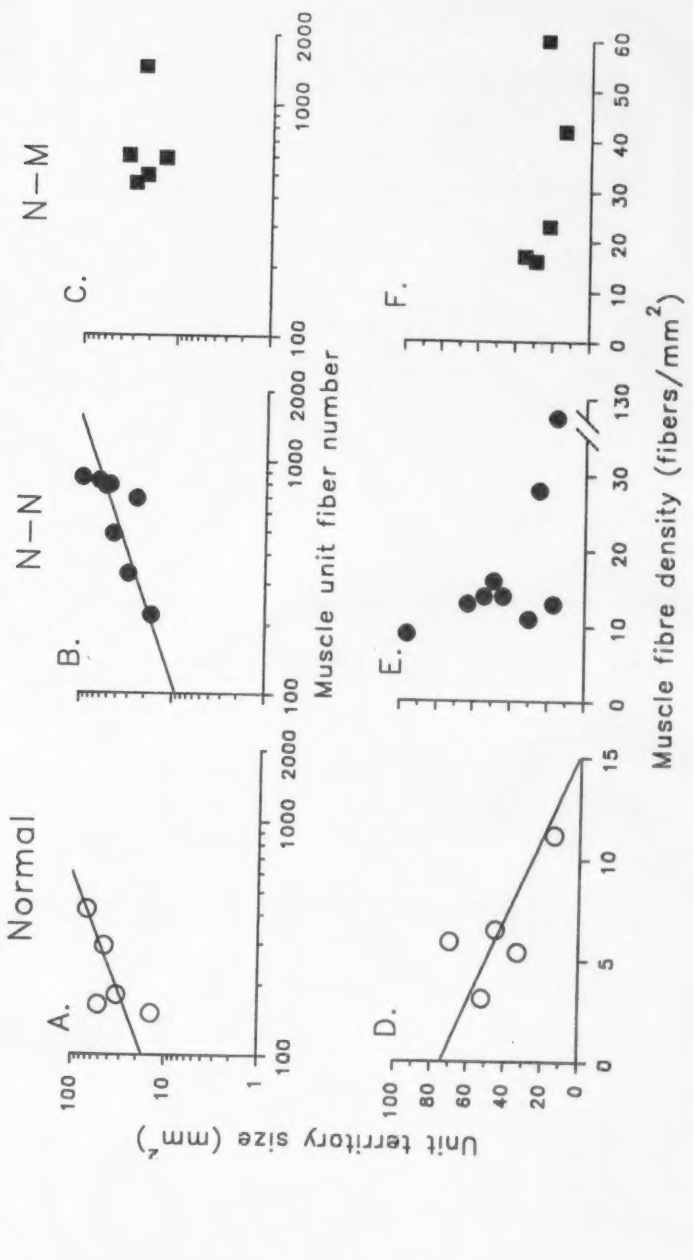

A
Control 1
151 mN
150 fibers
B
C
Control 2
185 mN
163 fibers
D
E
Control 5
528 mN
416 fibers
F

Figure 4.6.

Absolute unit territory size plotted as a function of muscle unit fiber number (A-C) and muscle fiber density (D-F) on double logarithmic and linear axes, respectively. MUs were isolated in 5 normal (A,D), 9 reinnervated muscles after N-N suture (B,E) and 5 reinnervated muscles after N-M suture (C,F).


Normal
N—N
N—M
A.
B.
C.
D.
E.
F.
Unit territory size (mm^2)
100
10
1
100
1000
2000
Muscle unit fiber number
100
80
60
40
20
0
0
5
10
15
20
30
130
40
50
60
Muscle fibre density (fibers/mm^2)

intermingled with non-unit fibers as shown in Fig. 4.4 and Table 4.3. Eighty percent of the fibers occurred as "singles" (not adjacent to another depleted fiber) and no more than 5 fibers are adjacent to each other within a single group. Chi-square analysis (see Methods for details) of the distribution of muscle unit fibers in areas of highest density within the muscle cross-section was used to determine whether the observed number of adjacencies between depleted fibers was significantly greater than would be expected if the fibers were randomly distributed. The Chi-square values for 3 normal MUs were 1.6, 3.8, and 4.1 (Table 4.3). Two of the 3 values were less than 3.84 indicating that the number of adjacencies between muscle unit fibers was not significantly different from random. The largest normal MU (Control #5; Table 4.3) had a significant Chi-square value (4.1) indicating that there were slightly more adjacencies than expected. A Chi-square value for the smallest S unit (Control #1; Table 4.3) could not be calculated using the 2 X 2 Chi-square contingency table (see Methods for details) since all muscle unit fibers occurred as singles. No adjacencies between muscle unit fibers implies that there are actually fewer adjacencies than expected if the distribution of fibers was completely random (Bodine et al. 1988). Interestingly, Bodine et al. (1988), using Chi-square analysis of muscle unit fiber distributions, also observed fewer adjacencies than expected in a S unit in the cat TA muscle.

4.3.3 Reinnervated muscle compartments

Reinnervated MUs were localized within regions along the longitudinal axis as observed in normal muscles (Table 4.1). Generally, reinnervated MUs either spanned the proximal third or distal third of the MG muscle. No glycogen-depleted MU was exclusively located in the region L1 as was observed for Control #1 (Table 4.1). Although not extensively investigated, this region tended to atrophy significantly more than the rest of the MG muscle when full muscle recovery was not observed. This trend was particularly noticeable in reinnervated muscles after N-M suture. Muscle fiber atrophy in the L1 region may result from few or no motor nerves branching into that region during reinnervation as a result of the compartmentalization (see Discussion).

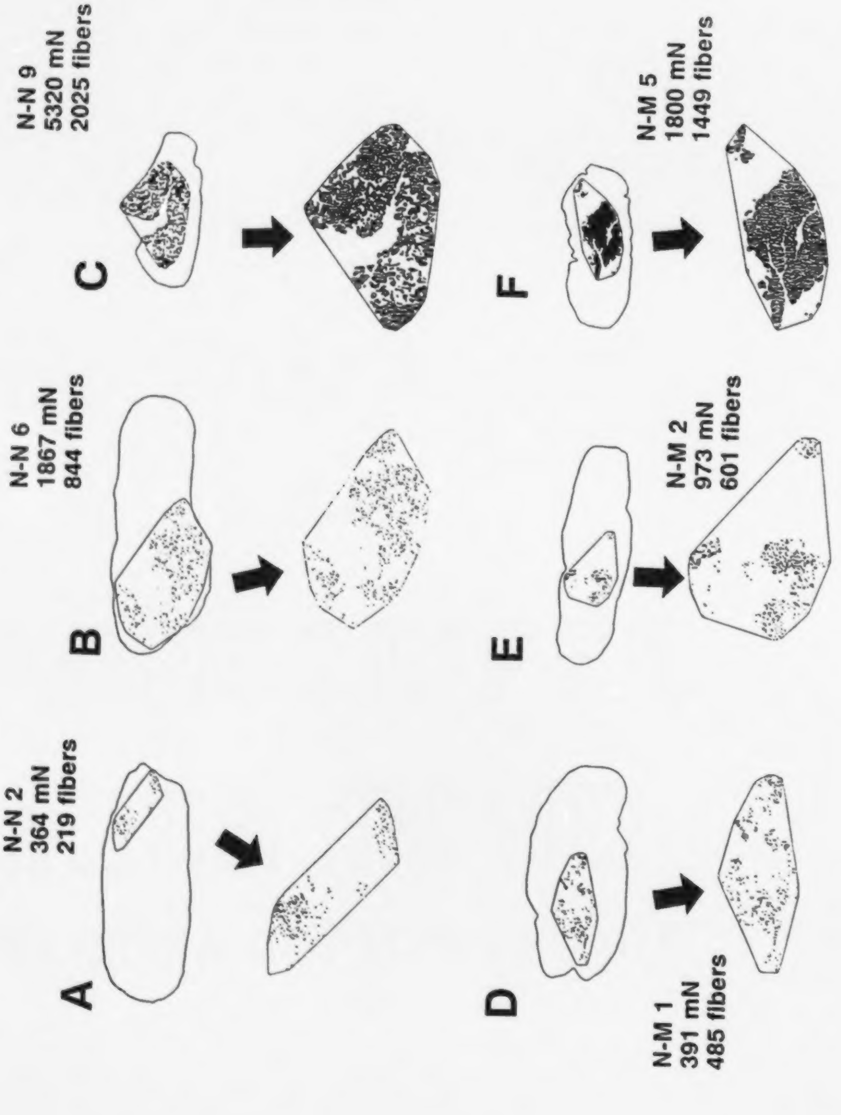
4.3.4 MU territory

Fig. 4.7 shows camera lucida drawings of 6 reinnervated MUs after N-N (Fig. 4.7A-C) or N-M sutures (Fig. 4.7D-F). As observed in normal muscles, reinnervated unit territories did not span across the entire muscle cross-section along the deep to superficial axis but were located either in the deep or superficial regions (Fig. 4.7, Table 4.1). The territory of 5 out of 14 reinnervated MUs did not span the entire muscle cross-section along the medial-ventral axis (Fig. 4.7, Table 4.1).

The size of the reinnervated unit territories increased with the size of the MU (number of muscle unit fibers) in muscles after N-N suture, but not after N-M suture, as shown in Fig. 4.6B and C, respectively. On average, unit territories were similar to normal in reinnervated muscles after N-N suture, but were significantly smaller than normal in muscles after N-M suture (Table 4.4). The density of muscle fibers tended to decrease with increasing size of reinnervated unit territory in muscles after N-N suture but showed little variation in muscles after N-M suture (Fig. 4.6E,F). The mean density

Figure 4.7.

Camera lucida drawings showing the location of the MU territory and distribution of depleted muscle unit fibers within the territory for 3 reinnervated MUs after N-N (A-C), or N-M sutures (D-F). Muscles are reinnervated by 62% (A), 32% (B), 2% (C), 66%, (D), 50% (E), and 4% (F) of their normal complement of MUs. All muscles have been drawn to the same scale to show the decrease in size as the number of innervating MUs is reduced and the generally smaller size of the muscles reinnervated after N-M suture. The drawings are oriented such that the top margin is lateral, the lower is medial and the left and right are deep and superficial, respectively.


A
N-N 2
364 mN
219 fibers
B
N-N 6
1867 mN
844 fibers
C
N-N 9
5320 mN
2025 fibers
D
N-M 1
391 mN
485 fibers
E
N-M 2
973 mN
601 fibers
F
N-M 5
1800 mN
1449 fibers

of muscle fibers was significantly higher than normal in reinnervated muscles (Table 4.4). The consequence of the branching patterns of regenerating nerves in establishing the size and density of the unit territory is discussed in detail in the Discussion.

Recovery of whole muscle CSA in reinnervated muscles was observed to be dependent on the number of MUs that reinnervated the MG and the type of nerve repair (ie. N-N or N-M suture) (Fig. 4.7). The muscles are drawn on the same scale in order to show the decrease in whole muscle CSA as the number of reinnervated MUs is reduced. The number of reinnervated MUs decreases from left to right in the figure. Whole muscle CSA falls dramatically when less than 20% MUs reinnervate the muscle after N-N suture and 66% after N-M suture (Table 4.2). The decreased muscle CSA is due in part to the reduced mean muscle fiber CSA which was 3946 μm^2 in normal muscles compared to 3149 μm^2 after reinnervation (Table 4.2). However, the dramatic decrease in muscle CSA cannot be solely accounted for by the decreased fiber size, but rather it is primarily caused by the dramatic reduction in total muscle fiber number (Table 4.2).

This is shown more clearly in Fig. 4.8A where the total muscle fiber number in a single muscle cross-section is plotted as a function of the number (%) of reinnervated MUs. The muscle fiber numbers in reinnervated muscle after N-M suture are not significantly reduced from normal unless fewer than 20% of the normal complement of MUs reinnervate the muscle. In contrast, the number of muscle fibers in reinnervated muscles after N-M suture is less than normal even when 50% of the MUs reinnervate the muscle. This result is consistent with the decrease in whole muscle force shown in

Figure 4.8.

Muscle fiber number, calculated as the ratio of the whole muscle fiber CSA and mean muscle fiber CSA measured in the muscle, plotted as a function of the number (%) of innervating MUs (A) in normal (open circles) and reinnervated muscles after N-N (open inverted triangles), N-M sutures (filled boxes). B. Recovery of whole muscle force, calculated as a percent of the force in the contralateral control muscle, plotted as a function of the number of reinnervated MUs. Values in B have been replotted from Fig. 3.2A in Chapter 3. Horizontal dashed lines represent mean fiber number in normal muscles (A) and muscle with 100% force recovery (B). Vertical lines represent the number of reinnervated MUs where recovery of force was incomplete after N-M suture.

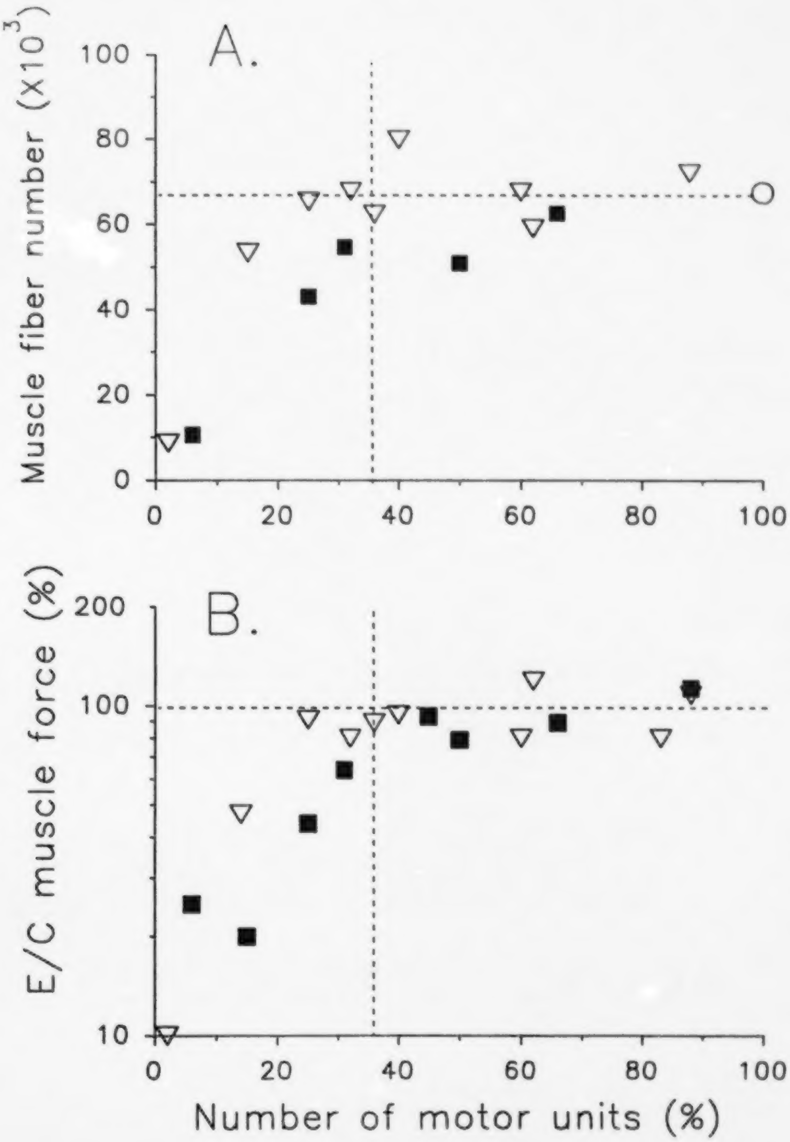

A.
Muscle fiber number (X10^3)
0
20
40
60
80
100
0
20
40
60
80
100
B.
E/C muscle force (%)
10
100
200
0
20
40
60
80
100
Number of motor units (%)

Table 4.4. Mean (± S.E.) values in normal and reinnervated muscles after N-N, and N-M sutures.

Condition	Normal	N-N	N-M
% MUs	100	44.5 ± 7.9	35.6 ± 10
Estimated Mean IR	215 ± 7.7	809 ± 191	487 ± 54
Unit Terr. (abs)	42.8 ± 8.4	43.8 ± 8.0	23.2 ± 3.0
Total Fiber CSA	0.93 ± 0.26	2.60 ± 0.37	1.6 ± 0.40
Density (fibers/mm^2)	6.4 ± 1.2	27.1 ± 12	31.6 ± 7.6
Adjacencies (80%)	1.0 ± 0.0	7.00 ± 1.2	20.0 ± 6.2
Max. Adj.	3.0 ± 0.57	25.0 ± 4.1	80.5 ± 25
Chi-square	3.2 ± 0.65	45.1 ± 10	158 ± 27

Abbreviations same as Tables 1-3.

Chapter 3 and replotted in Fig. 4.8B for comparison. Since denervated fibers were not evident in the muscle following long-term reinnervation ($\geq$4 months) the fibers not reinnervated by regenerating nerves presumably degenerated and were not visible.

Muscle unit fiber clumping was not as dramatic in reinnervated cat MG muscles (Fig. 4.7A,D) as compared to the extensive clumping observed in reinnervated rat TA where the majority of MUs presumably reinnervated the denervated muscle (Brandstater and Lambert, 1973; Kugelberg et al. 1970; Tötösy de Zepetnek et al. 1992). Visually, the extent of clumping increased in reinnervated muscles after both N-N (Fig. 4.7A-C) and N-M (Fig. 4.7D-F) sutures when fewer MUs reinnervated the muscle (compare from left to right in Fig. 4.7). As observed in normal muscles (Fig. 4.5), there were also regions of high and low densities of muscle unit fibers within the unit territory. The highly clumped regions are presumably the result of extensive terminal sprouts which reinnervated muscle fibers within a small localized region (Gordon et al. 1991; Kugelberg et al. 1970; Tötösy de Zepetnek 1990). However, the relatively large distances between clumped regions suggests that the regenerating motor axon also made at least a few proximal branches along the length of the main nerve branches.

The number of adjacencies between muscle unit fibers, and Chi-square values were all significantly higher in reinnervated cat MG muscles compared to normal (Tables 3,4) and significantly higher than would be expected if they were randomly distributed. On average, Chi-square values and number of adjacencies were both significantly higher in muscles after N-M as compared to N-N suture (Table 4.4) indicating that the extent of muscle unit fiber clumping was more extensive after N-M than N-N suture.

Figure 4.9.

Chi-square values plotted as a function of the number (% of normal) of reinnervating MUs in muscle after N-N (A) or N-M sutures (B). The slope of the regression line in B is significantly steeper than the line in A and both are significantly different from zero ($p < 0.05$). The slope (± S.E.) of the regression lines in A and B are: -0.78 ± 0.18 (RO: 0.84), and -4.6 ± 0.86 (RO: 0.84), respectively.

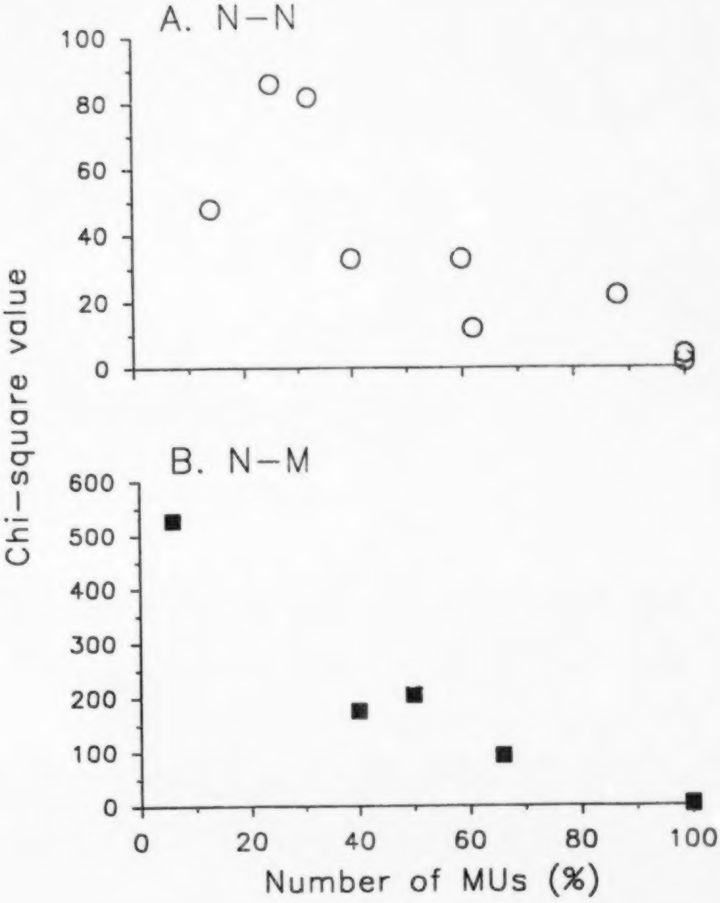
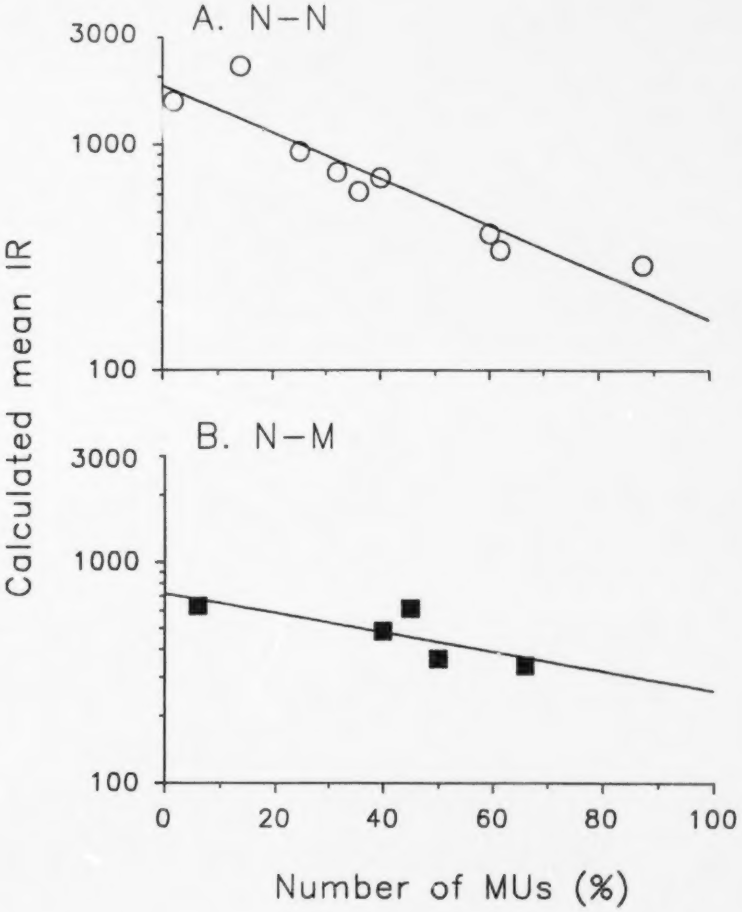

A. N–N
B. N–M
Chi–square value
Number of MUs (%)
0
20
40
60
80
100
0
100
200
300
400
500
600

Figure 4.10.

Calculated mean IR of MUs in reinnervated muscles after N-N (A) or N-M sutures (B) plotted as a function of the number of innervating MUs (% of normal). Mean IR determined by dividing estimated number of fibers in a single cross-section by the estimated number of reinnervated MUs (see Methods for details). Mean IR increases inversely with the number of reinnervated MUs in both conditions, however the increase is significantly greater after N-N suture. The slopes (± S.E.) of the regression lines in A and B are -0.011 ± 0.002 (RO: 0.91) and -0.007 ± 0.002 (RO: 0.92), respectively, and are both significantly different from zero ($p < 0.05$).


A. N–N
B. N–M
Calculated mean IR
3000
1000
100
3000
1000
100
0
20
40
60
80
100
Number of MUs (%)

When Chi-square values are used as an index of muscle unit fiber clumping the extent of clumping increased inversely with the number (%) of reinnervated MUs as shown in Fig. 4.9. The higher Chi-square values for a similar number of reinnervated MUs in muscles after N-M (Fig. 4.9B) as compared to N-N sutures (Fig. 4.9A) indicates that there is a greater tendency for muscle unit fibers to be clumped after N-M suture.

4.3.5 IR of normal and reinnervated MUs

The mean unit IR for each muscle was estimated by dividing the total number of muscle fibers in a single transverse muscle cross-section by the estimated number of MUs in the muscle. Using this calculation the mean unit IR, in normal muscles, was determined to be 215 ± 7.7 fibers (Table 4.4). Direct counts of glycogen-depleted fibers in the five normal muscles (Table 4.1) gave a mean IR of 264 ± 61 fibers. The relatively good agreement between the two mean IR values shows that the mean IR, calculated as the ratio of muscle fiber and MU numbers, is a reasonably accurate measure of mean IR.

Following reinnervation the mean IR increased significantly from normal when few MUs reinnervated the muscle (Table 4.4). As shown in Fig. 4.10, the IR increased inversely with the number of reinnervated MUs. The steeper slope of the regression line fitted through the N-N, compared to N-M values, indicates that MUs can increase in size by a greater extent when the nerve is sutured to its distal stump (N-N suture) as compared to when it is sewn directly to the muscle fascia (N-M suture). The smaller

Figure 4.11.

Whole muscle cross-sections stained for myosin ATPase with acid preincubation (except B, with alkaline preincubation) from normal muscles (A), and muscles reinnervated by 88% (B), 36% (C), 14% (D), and 2% (E) of their normal complement of MUs after N-N suture.

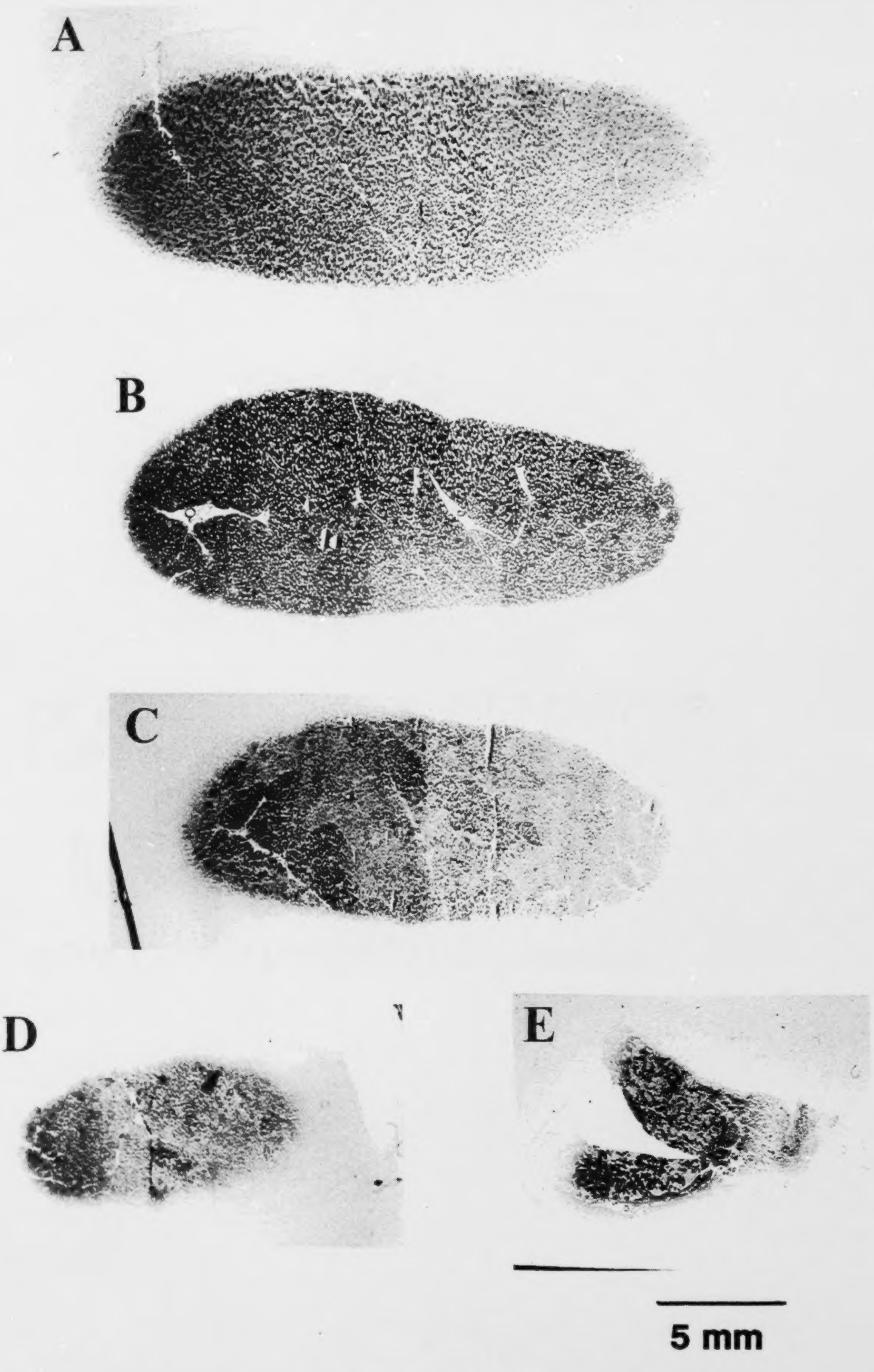
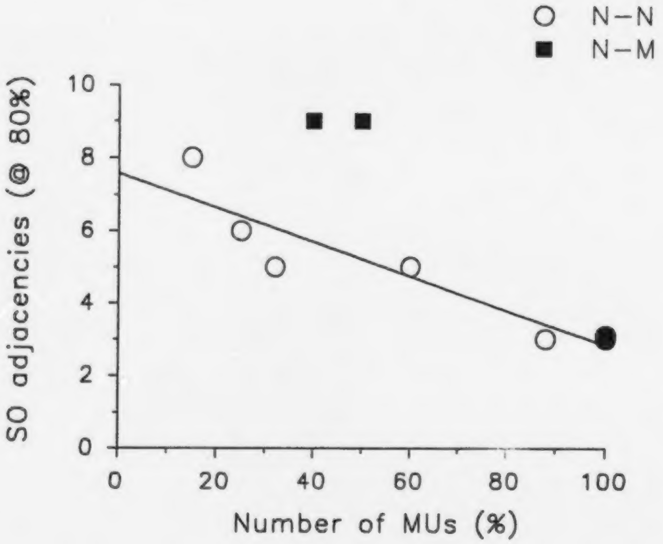

A
B
C
D
E
5 mm

Figure 4.12.

The extent of SO fiber grouping, as determined by the number of adjacencies between SO fibers @ 80%, plotted as a function of the number of reinnervating MUs (% of normal) in 2 normal muscles (filled circles), 6 reinnervated muscles after N-N suture (open circles) and 2 reinnervated muscles after N-M suture (filled boxes). The extent of SO fiber grouping in reinnervated muscles after N-N suture increases inversely with the number of reinnervated MUs as indicated by the significant slope of the regression line fitted to the values (slope ± S.E.: -0.05 ± 0.01, RO: 0.93). SO fibers were substantially more grouped when a similar number of MUs reinnervated the muscle after N-M suture as compared to N-N suture (compare filled boxes and open circles).


○ N−N
■ N−M
SO adjacencies (@ 80%)
0
2
4
6
8
10
0
20
40
60
80
100
Number of MUs (%)

increase in mean IR in reinnervated muscles after N-M suture accounts for both the smaller increase in mean unit force (Chapter 3) and loss of muscle fibers when few MUs reinnervated the muscle (Table 4.2, Fig. 4.8A).

4.3.6 Muscle fiber type-grouping

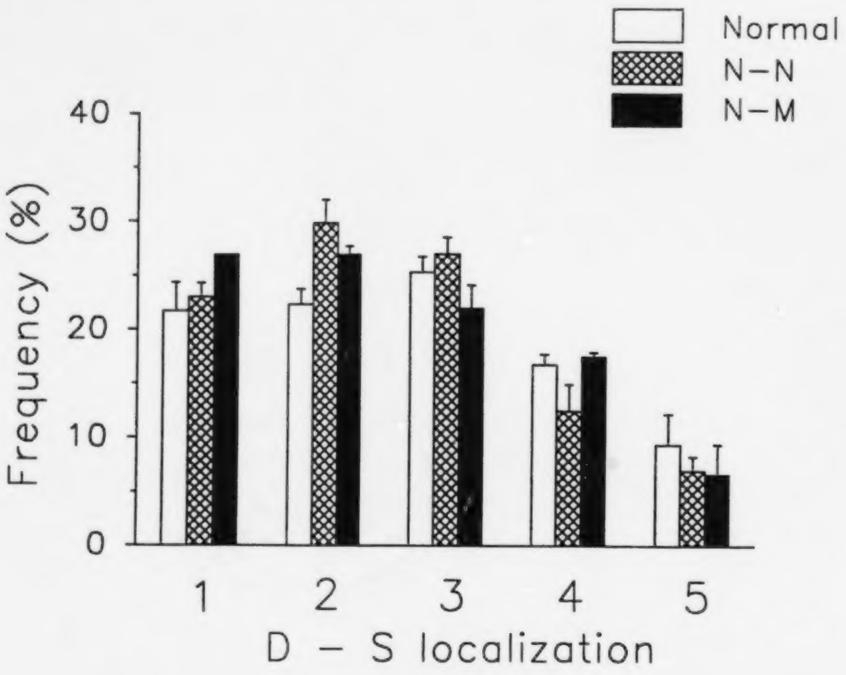
Fig. 4.11 shows the distribution of the different fiber types in normal and reinnervated cat MG muscles. Reinnervated muscles in Fig. 4.11 are innervated by progressively fewer MUs (B-E). In normal muscles, SO fibers are typically grouped together in clusters of 1-5 fibers (Table 4.3). Following reinnervation the aggregation of SO fibers generally increased (compare Fig. 4.11A and C), however the severity of type-grouping was not the same for all reinnervated muscles. Muscles in Fig. 4.11B-E are reinnervated by progressively fewer MUs after N-N suture. As was observed with the clumping of fibers within a single MU, the extent of type-grouping depended on how many motor nerves reinnervated the muscle and the type of nerve repair. As shown in Fig. 4.12 the grouping of SO fibers increased inversely with the number (%) of reinnervated MUs after N-N suture (open symbols). When nearly all MUs (88%) reinnervated the muscle, 80% of the SO fibers occurred in groups of three or less which is very similar to normal. As the number of MUs was reduced the grouping of SO fibers increased significantly. Grouping of SO fibers was significantly higher in muscles after N-M as compared to N-N suture when a similar number of MUs reinnervated the muscle as indicated by the filled squares in Fig. 4.12. SO fibers were presumably grouped together in reinnervated muscles after N-M suture, compared to N-N suture, because the muscle fibers within single MUs were significantly more clumped (Fig. 4.9, Table 4.3).

4.3.7 Regionalization of muscle fiber types following reinnervation

The distribution of fiber types in the MG muscle is highly regionalized with the superficial region being predominantly composed of FOG and FG fibers while the deep region is highly mixed with all 3 fiber types (ie. SO,FOG,FG). This is shown for 1 normal muscle stained for myosin ATPase in Fig. 4.11A. Regionalization of SO fibers was observed in all experimental muscles reinnervated by >25% of the normal complement of MUs (Fig. 4.11B,C). This is quantified in Fig. 4.13 which compares the mean (± S.E.) number of SO fibers, calculated as a percent of the total number counted, in 5 areas (D1-D5) located from the deep to superficial regions (see Methods), in normal muscles and muscles reinnervated by <25% of their MUs after N-N and N-M sutures. When less than 15% of the MUs reinnervate the denervated muscle the regionalization of muscle fiber types is lost as shown for 2 muscles in Fig. 4.11D,E (14% and 2% of the normal complement of MUs, respectively).

Figure 4.13.

Bar graph showing the mean (± S.E.) number of SO fibers, calculated as a percent of the total number counted, in 5 different areas located from the deep to superficial regions of the muscles (Fig. 4.2B), from 5 normal (open bars), 5 reinnervated muscles after N-N (cross-hatched bars), and 3 muscles after N-M suture (filled bars). All muscles were innervated by >25% of the normal complement of MUs. SO fibers are regionalized in both normal and reinnervated muscles containing >25% of their MUs such that the superficial regions (4,5) contain fewer SO fibers.


Normal
N-N
N-M
Frequency (%)
0
10
20
30
40
1
2
3
4
5
D - S localization

4.4 DISCUSSION

Using glycogen depletion to analyze fiber number and location of MUs in the large cat MG muscle under normal conditions, and conditions where regenerated MUs are forced to enlarge, we have obtained clear evidence for compartmentalization in normal MG muscles to support previous findings (Letbetter 1974; Weeks and English 1987) and that regeneration restores compartmentalization. Our results indicate that compartmentalization arises because axons follow major nerve trunks before branching to establish MU territories in the compartments. Proximal branching can explain territory size and more distal branching within this territory is the mechanism by which nerves reinnervate more muscle fibers than normal to compensate for the reduced number of reinnervated MUs. The muscle unit fibers become progressively more clumped within the territory and the size of the territory ultimately limits the reinnervated MU size. Type-grouping accurately reflects enlargement of MUs as it parallels muscle unit fiber clumping in large muscles such as the cat MG.

4.4.1 Spatial analysis of glycogen-depleted muscle unit fibers

The accuracy of counting and analyzing the number (IR) and distribution of glycogen-depleted fibers from a single MU depends on the muscle architecture including muscle fiber length and pinnation angle. For example, the muscle fibers of the cat (Bodine et al. 1987), and rat TA muscles (Tötösy de Zepetnek et al. 1992), have relatively small pinnation angles and the fibers are long extending for more than 50%

of the muscle length. Consequently, a single muscle cross-section with the highest number of depleted muscle unit fibers provides an accurate estimate of the total number of muscle fibers (IR) within a single depleted MU. The cat MG muscle is a more complex muscle such that the fibers are shorter and the angle of pinnation is larger (Burke and Tsairis 1973). As a result, a single cross-section containing the maximum number of depleted muscle unit fibers represents only 50 to 67% of the total number of fibers (Burke and Tsairis 1973). It is therefore important to determine whether the number and distribution of muscle fibers in the MG muscle cross-section containing the maximum number of depleted fibers provides a reasonable estimate of the unit IR and fiber distribution.

Several serial sections were made from each of five muscle blocks along the longitudinal axis to locate the cross-section containing the maximum number of glycogen-depleted muscle unit fibers (Table 4.1, Fig. 4.4). As previously noted by Burke and Tsairis (1973), one whole muscle cross-section always contained the highest number of glycogen depleted fibers belonging to a single MU. If it is assumed that each MU territory spans obliquely across the longitudinal axis of the muscle then the cross-section with the highest number of muscle unit fibers represents a fixed percentage of the true unit IR (Burke and Tsairis 1973). This assumption is supported by the fact that there is a good correlation between the number of glycogen-depleted fibers counted in the cross-section containing the highest number of muscle unit fibers and unit tetanic force (Fig. 4.12, Chapter 3), a relationship that was found in cat and rat TA muscles where all muscle unit fibers could be counted due to the small angle of pinnation (Bodine et al. 1987; Tötösy de Zepetnek et al. 1992). The position of muscle fibers may be displaced if the angle of pinnation between different fibers vary. However, since all fibers in the cat MG essentially form unipinnate bands extending from an aponeurosis of origin to an aponeurosis of insertion (English and Letbetter 1982) the relative location of each fiber should be the same in each whole muscle cross-section. This is particularly valid for fibers located in the central region of the unit territory. As a result, we are confident that an analysis of the depleted fibers in the cross-section containing the largest number of MUs gives a reasonable estimate of unit IR and we can compare the distribution and territories of MUs from different muscles.

4.4.2 MU distribution and muscle compartments

Our spatial analysis of MU distribution in normal MG provides strong evidence for compartmentalization of the MG muscle by the major nerve branches (Letbetter 1974; Weeks and English 1987). Muscle unit fibers were located in at least two regions along the longitudinal axis of the muscle (Table 4.1). For example, one MU was located exclusively in the most proximal region of the muscle (L1, Table 4.1) while two spanned the proximal third (L1,L2,L3, Table 4.1). Along the deep to superficial axis, muscle unit fibers were primarily located either in the deep or superficial portion of the muscles (Fig. 4.5; Table 4.1). These data indicate that MG muscle unit fibers do not span through the entire muscle (see also Burke and Tsairis 1973), but rather are located within neuromuscular compartments as originally suggested by Letbetter (1974) on the basis of microdissection of the MG nerve along with glycogen-depletion techniques. As shown by Weeks and English (1987), the MG nerve bifurcates into two primary branches. One branch innervates fibers in the most proximal region of the muscle while the second branch extends distally. The distal nerve subsequently divides into four branches to innervate deep and superficial fibers in the muscle. Our spatial analysis of the location of MUs in normal MG muscles provides independent evidence for this compartmentalization: normal MUs were localized in neuromuscular compartments innervated by motor axons that either branched 1) proximally (Control #1, Table 4.1), 2) distal and superficially (Control #5), or 3) distal and ventrally (ie. deep region, Control #4).

4.4.3 MU territories and nerve branching

The absolute size of normal MU territories, which ranged from 14 to 70 mm^2 (Table 4.2), is similar to that in the cat TA muscle (Bodine-Fowler et al. 1990) and both are significantly larger than the those found in the smaller TA muscles of the rat reported by Tötösy de Zepetnek (1990) (range: 3.68 to 22.6 mm^2). In contrast, if territory size is expressed as a percentage of whole muscle CSA the mean size in all muscles is very similar: 15% (cat TA: Bodine et al. 1987), 17% (rat TA: Edstrom and Kugelberg 1968), 21% (rat TA: Tötösy de Zepetnek 1990) and 20% (cat MG: present study). The similarity in the relative territory size in different muscles, varying dramatically in size, shows that the expanse of axon branching establishes MU territory size proportionately to the size of the muscle.

Unit territory size also depend on IR, increasing as the number of muscle unit fibers increased (Fig. 4.6A) in agreement with the results of Bodine-Fowler et al. (1990) in the cat TA and by Tötösy de Zepetnek (1990) in rat TA muscles. Since unit force correlates well with electrophysiological measures of axon diameter (Appelberg and Emonet-Denand 1967; Gordon and Stein 1982; Wuerker et al. 1965) and IR varies systematically with unit force (Chapter 3; Bodine et al. 1987; Tötösy de Zepetnek et al. 1992) these results suggest that the size of the unit territory and IR in normal muscles varies as a function of axon size.

Visually, the distribution of normal cat MG muscle unit fibers appears random or scattered throughout the unit territory (Fig. 4.5; see also Burke and Tsairis 1973). However, the muscle fibers are not uniformly distributed throughout their territory as indicated by areas of high and low densities (Bodine-Fowler et al. 1990). The regions of high density may result from localized branching near the terminal of the motor axon while the larger distances between fibers is the result of more proximal branching in the nerve trunk as suggested by Kugelberg et al (1970). Similarly, muscle unit fibers that are adjacent to each other may reflect very local terminal branching of the motor axon. The low occurrence of adjacent muscle unit fibers in normal muscles, as determined by both the frequency of adjacencies and Chi-square analysis, may be interpreted that most of the branching occurs proximally rather than near the endplates.

4.4.4 Reinnervated muscle compartments

Reinnervated MUs are located in the same muscle compartments as normal. These findings suggest that regenerating axons branch within the same nerve trunks which define the normal muscle compartments. Reinnervated muscle unit fibers were generally located within the proximal or distal third of the MG muscle along its longitudinal axis (Table 4.1) and in the deep or superficial regions in the transverse axis of the muscle (Fig. 4.7., Table 4.1). When we forced the nerves to grow along the muscle fibers (N-M suture) the muscles were also compartmentalized presumably because the regenerating nerves entered the sheaths at some point. The finding that compartments are reestablished after N-M suture provides good evidence that regenerating nerves are strongly attracted to denervated nerve sheaths as has been shown previously in frog muscle (Kuffler 1986; Pecot-Dechavassine and Diaz 1991).

A frequent observation was that the most proximal region of the reinnervated muscles was often severely atrophied, especially when the number of reinnervated MUs was low and the nerve was sutured directly to the muscle (N-M suture). According to the observations of Weeks and English (1987) the separation of the most proximal branch from the distal branches occurred very proximal to the nerve entry into the muscle. In addition, the nerve branch was relatively small. It is therefore conceivable that when the number of regenerating nerves is severely reduced the majority of the remaining motor axons simply elongate along the distal nerve sheaths and bypass the small proximal nerve. As a result, the muscle fibers in the proximal regions remain denervated.

4.4.5 Reinnervated MU territory and nerve branching

When nerves regenerate within intramuscular nerve sheaths after N-N suture we found that the absolute MU territory size was the same as normal (Fig. 4.6, Table 4.4). This result strongly implies that the expanse of proximal branching in the nerve trunks was as great as normal. However, the clumping of reinnervated muscle unit fibers within the territory indicates more extensive terminal branching (Kugelberg et al. 1970; Gordon et al. 1991). If regenerating axons are forced to reinnervate more muscle fibers than normal, by reducing the MU number, it does so by increasing the number of distal, and not proximal branches, because fibers become progressively more clumped as the number of reinnervated MUs is reduced (Fig. 4.9). After N-M suture, the proximal nerve branching is reduced and consequently the MU territory size is smaller. As a result, there is even more extensive clumping of muscle unit fibers within the territory as compared with N-N suture: both with respect to the number of adjacencies between depleted fibers (Table 4.4) and the calculated Chi-square values (Fig. 4.9, Table 4.4).

The calculated mean IR increased inversely with the number of reinnervated MUs after N-N, and N-M sutures (Fig. 4.10). This increase in IR indicates that the reinnervated MUs increased in size to compensate for the reduced MU numbers. However, as was the case for mean unit tetanic force (Chapter 3), the increase in IR was significantly higher after N-N suture compared to N-M suture. The greater capacity for MUs to enlarge after N-N suture appears to be related to the size of the unit territory. A larger territory simply provides the regenerating axon with a greater number of muscle fibers in which to innervate.

Thus, MU territory size ultimately limits the extent of distal branching and therefore MU size. When there are fewer than 15% of the normal complement of MUs, all fibers within a single MU are clumped (Fig. 4.7C) with the result that MUs cannot enlarge further. When MU territory size is reduced after N-M suture, MU size is more severely restricted since the number of available muscle fibers within the territory is smaller. Since the MU territory size cannot increase by terminal branching, presumably due to epimysial barriers (Kugelberg et al. 1970), many muscle fibers remain denervated and muscles recovery is severely compromised (Fig. 4.8).

The size of the reinnervated unit territories may also explain why clumping of muscle unit fibers is so severe in reinnervated rat TA muscles (Kugelberg et al. 1970; Tötösy de Zepetnek et al. 1992). The mean size of the unit territories of reinnervated rat MUs is only 37% that of normal (Tötösy de Zepetnek 1990). This decrease in territory size is significantly greater than that observed for reinnervated cat MG units in muscles after either N-N or N-M suture (Fig. 4.6, Table 4.4). As was the case with the smaller territories in muscles with N-M sutures the reinnervating motor axons in rat TA are forced to innervate fibers within an extremely confined area resulting in a dramatic increase in clumping between fibers within the same MU. Why are reinnervated unit territories smaller in rat TA muscles as compared to cat MG? One possible explanation is that the normally greater branching of MUs in the larger cat MG muscle promotes more branching during regeneration. For example, the mean IR is 4 times larger in the cat MG muscle (ie. 528 fibers; Chapter 3) compared to the smaller rat TA msucle (ie. 121 fibers; Tötösy de Zepetnek et al. 1990). It is possible that each branch point along the intramuscular endoneurial nerve sheath acts as a potential decision point for the regenerating nerves to branch. If factors promoting axon-axon adhesion (eg. L1;

Rathyen et al. 1987; Lemon et al. 1989) is stronger than factors promoting axon-muscle adhesion (eg. N-CAM; Covault et al. 1985) during regeneration then axons will presumably branch less often then normal. Therefore, given a finite probability of branching at each point, it follows that regenerating axons confronted with more branch points, as in larger muscles, will branch more often and more extensively than those with fewer branching points. Since normal MUs in small muscles, such as the rat TA, do not branch as extensively as in larger muscles the regenerating MU territories are smaller. Once the size of the territory is established, MUs must branch locally (terminal branching) within the territory in order to increase in size. Consequently smaller territories results in greater muscle unit fiber clumping.

4.4.6 Muscle fiber type-grouping

Type-grouping paralleled the clumping of MU fibers when the reinnervated MU territory size was similar to normal after N-N suture such that type-grouping increased as MU numbers decreased. Thus, under conditions where proximal branching reestablished MU territory size, type-grouping reflected the number of reinnervated MUs. These results are consistent with the proposal of Karparti and Engel (1968) that type-grouping depends on the severity of the nerve injury which in turn governs the number of regenerating motor axons. When the unit territory is reduced, as after N-M suture, type-grouping was substantially greater, as compared to N-N suture, for the same number of reinnervated MUs (Fig. 4.12). Therefore, type-grouping can reflect both a decreased MU territory size due to less proximal branching and increased MU size due to more distal nerve branching. The relative absence of type grouping observed by Gordon et al. (1988) and Nemeth et al. (1992) in the cat MG muscle following cross- and self-reinnervation, respectively, may be explained if a large number of MUs reinnervated the denervated muscles. The severity of fiber type grouping commonly reported in reinnervated cat muscles (Dum et al. 1985a; Foehring et al. 1986a) and smaller muscles of the rat (Gillespie et al. 1986; Karparti et al. 1968; Kugelberg et al. 1970; Parry and Wilkinson 1990; Tötösy de Zepetnek et al. 1992) may reflect a small number of reinnervated MUs or reduced proximal branching which is not associated with a decrease in MU numbers.

4.4.7 Regionalization

Normally the cat MG muscle is composed of 3 different muscle fiber types which are regionalized throughout the muscle cross-section. The superficial region predominantly contains FG and FOG fibers while the deep region is composed of all 3 fiber types (Fig. 4.11). Following reinnervation, after either N-N or N-M suture, the normal regionalization of muscle fiber types was reestablished in the cat MG muscle despite a reduction of up to 80% of the MUs and significant type-grouping (Fig. 4.13). With fewer than 20%, regionalization of different fiber types was lost (Fig. 4.11D). Regionalization of fiber types after reinnervation has also been reported in rat TA (Parry and Wilkinson 1990; Tötösy de Zepetnek et al. 1992) and cat LG muscles (Foehring et al. 1987). Regionalization can occur in reinnervated muscle if 1) motoneurons selectively reinnervate their original muscle fiber types (McLennan 1983; Thompson et al. 1987) 2) motor axons topographically reinnervate muscle fibers along the muscle cross-section in a similar manner as during development (Brown and Everett 1990; Brown and Everett 1991; Laskowski and Sanes 1988) 3) certain reinnervated muscle fiber types are resistant to histological respecification by the reinnervated axon (Chan et al 1982; Dum et al 1985b; Foehring et al 1987; Gillespie et al 1986; Parry and Wilkinson 1990) possibly due to intrinsic differences established by clonal development (Miller and Stockdale 1986) or 4) differential mechanical loading of muscle fibers in different regions contributes to the expression of one phenotype type over another (Gordon and Pattullo 1992). Although none of the four possibilities can be totally ruled out from the results of the present study, the first seems unlikely since the dramatic clumping of muscle unit fibers, along with the presence of fiber type-grouping, argues against selective reinnervation of regenerating motor axons to their original fiber types. In addition, several studies have also shown that regenerating motor axons do not show any specificity for their original muscles (Bernstein and Guth 1961; Miledi and Stefani 1969; Thomas et al. 1987; Weis and Hoag 1946) even when two muscles are reinnervated by the same nerve (Gillespie et al. 1986). Recent experiments in our lab have shown that the normal regionalization of fiber types in cross-reinnervated rat TA is reestablished even when the tibial nerve is sutured to the TA muscle opposite to the side where the common peroneal nerve normally enters (Fu et al. 1992). These results argue against topographical reinnervation of muscle fibers.

In summary, the results from this study indicate that reinnervated muscle unit fibers are more clumped than normal, but significantly less than that observed in smaller murine muscles. The extent of clumping of both muscle unit fibers and fiber types was inversely related to the number of reinnervated MUs. Finally, the extent of clumping appears to be related to the size of the unit territory which in turn in governed by the number of proximal branches of the regenerating motor axon. Type-grouping reflects muscle unit fiber clumping. In large muscles, type-grouping reflects a decreased number of reinnervated MUs and MU enlargement and may not be present following reinnervation per se if the number of MUs is similar to normal. In smaller muscles, however, type-grouping is diagnostic of reinnervation as well as reduced MU numbers.

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5. MOTOR UNIT PROPERTIES IN PARTIALLY DENERVATED AND REINNERVATED CAT MG MUSCLES

5.1 INTRODUCTION

Normally, unit tetanic force is directly related to electrophysiological measurements of axon size, such as axon conduction velocity (Appelberg and Emonet-Denand 1967; McPhedran et al. 1965; Wuerker et al. 1965) or axon potential amplitude (Gordon and Stein 1982a; Gordon et al. 1986). Unit tetanic force is inversely related to twitch contraction time such that the largest force producing motor units (MUs) have the fastest contractile speed (Bagust et al. 1973; Gordon and Stein, 1982a; Stephens and Stuart, 1975; Wuerker et al. 1965). These size relationships return after both self- (Bagust and Lewis, 1974; Foehring et al 1986a; Gordon and Stein 1982a; Tötösy de Zepetnek et al. 1992b) and cross-reinnervation (Foehring et al. 1987a; Gordon et al. 1986, 1988) even though the normal differences in contractile properties between MU types is not as obvious (Foehring et al. 1986b; Gordon and Stein 1982b; Gordon et al. 1986;1988).

Since regenerating nerves do not innervate their original muscles (Kugelberg et al. 1970; Tötösy de Zepetnek et al. 1992a), and the return of the normal size relationships is time dependent (Gordon and Stein 1982ab), it has been argued that size relationships are reestablished because the nerve respecifies the reinnervated muscle fiber properties (Gordon and Stein 1982; Gordon et al. 1988). However, recent experiments have shown that the number of muscle fibers innervated by a single motoneuron (innervation ratio; IR) is an important factor determining the range in unit force in normal (Bodine et al. 1987; Chamberlain and Lewis 1989; Tötösy de Zepetnek et al. 1992a; and Fig. 6.8) and reinnervated muscles (Tötösy de Zepetnek et al. 1992a; and Fig. 3.12). Even when IR increased by sprouting in partially denervated muscles, where differences between fiber types were lost (see Chapter 2 for details), the normal size relationships returned. Thus, size dependent branching is an important determinant of force in normal, regenerating and sprouting motor nerves.

In contrast to these studies, Lewis and colleagues (Lewis et al. 1982) indicated that, following cross-reinnervation of the cat soleus with nerves supplying either the flexor hallucis (FHL) or flexor digitorum longus (FDL) muscles, unit tetanic force was inversely related to conduction velocity while twitch contraction was directly related. Both size relationships are the reverse of that observed in normal muscles. One factor suggested by Lewis and coworkers which contributed to these inverse relationships was that the smallest motor nerves reinnervated a larger number of muscle fibers as compared to larger nerves (Lewis et al. 1982). Since only ~65% of the FHL motoneurons reinnervated the denervated soleus muscle (Lewis et al. 1982) it may be possible that under conditions where fewer MUs reinnervate the muscle, small motor axons branch more extensively and reinnervate a larger number of muscle fibers which consequently results in a loss of the normal size relationships.

After the initial cross-reinnervation experiments of Buller, Eccles, and Eccles (1960) showed that whole muscle contractile properties are modulated by the reinnervating nerve, numerous studies have since shown that contractile and histological properties of reinnervated motor units are changed by the reinnervating nerve (Bagust et al. 1981; Chan et al. 1982; Dum et al.1985ab; Foehring et al. 1987a; Gillespie et al. 1986; Gordon and Stein 1982ab; Gordon et al. 1988). However, several lines of evidence suggest that muscle fiber properties may not be entirely neuron regulated. These include an increase in reinnervated MUs with intermediate fatigue characteristics (Gordon et al. 1986), difficulty in classifying reinnervated MUs on the basis of sag (Gordon et al. 1986), incomplete conversion of soleus muscle fibers after cross-reinnervation with nerves formerly supplying fast muscle fibers (Foehring et al 1987b. Dum et al. 1985b), and poor respecification of muscle fiber size by the reinnervating nerve (Tötösy de Zepetnek et al. 1992a).

In the present study, we characterized a large sample of MUs in reinnervated muscles where the number of regenerating nerves was experimentally reduced to force MUs to increase their IR to compensate for the reduced MU number. By studying MU contractile properties of force, speed, fatigability and recording axon potential amplitude, we have first readdressed the issue as to what extent nerves respecify muscle fiber contractile properties. Second, we have investigated whether the normal size relationships are reestablished when the number of reinnervating MUs is dramatically reduced and the motor nerve is forced to enlarge and reinnervate muscle fibers that formerly belonged to different MUs. Third, we examined these questions under experimental conditions where we have previously shown that MU enlargement is severely restricted. Finally, in several experiments muscle fibers from a single MU were depleted of glycogen in order that they could be identified histochemically to determine whether regenerating nerves respecify muscle fiber size.

5.2 METHODS

A total of 44 cats of both sexes (21 females, 23 males), with a mean (± S.E.) weight of 3.2 ± 0.08 kg, were used in this study. Six of the cats were unoperated, control animals. Thirty-eight cats received an initial surgery between 3.0 to 18 months (mean ± S.E.: 6.8 ± 0.7 months) prior to the final acute experiment. The MG muscle was either partially denervated (10 cats) or completely denervated by 1) crushing the MG nerve (7 cats) or by 2) sectioning the nerve and suturing it to the distal stump (N-N suture; 11 cats) or 3) suturing it directly to the muscle fascia (N-M suture; 10 cats). In all completely denervated MG muscles ($n=28$) the number of motor axons reinnervating the muscle was simultaneously reduced by sectioning 1 of the 2 spinal roots (L7 and S1) that supplies nerves to the triceps surae muscles of the cat. Of the 38 experimental cats MG units were isolated and characterized in 35 animals.

Animal surgery, MU recording, quantification of reinnervated MU numbers, and histochemical analysis have been described in detail in the preceding Chapters.

5.2.1 Statistical analysis

Throughout this paper arithmetic means are given with standard errors (S.E.). Statistical difference between 2 means was determined using the Students t-test. Regression lines were fitted according to the least mean squares criterion (Hartley, 1961) and are only drawn through the data points if they are significantly different from 0 at the 0.95% confidence level.

Data from different animals were pooled together only if the distribution of the parameters measured was not significantly different from each other as determined by the nonparametric Kruskal-Wallis test (Sokjal and Rohlf, 1969). The pooled data were collected by using logarithmic bin intervals to represent the logarithmic distribution of force values. The unit tetanic force on the X-axis is shown in logarithmic intervals of equal bin width and not of the exponential values used to calculate the bin intervals in order that the actual force values can be more readily visualized (Figs. 5.7 and 5.9). Significant differences between cumulative distributions were tested for by applying the Kolmogrov-Smirnov test (Fisz 1963). Significant differences between cumulative distributions were tested for by applying the Kolmogrov-Smirnov test (Fisz, 1963).

5.3 RESULTS

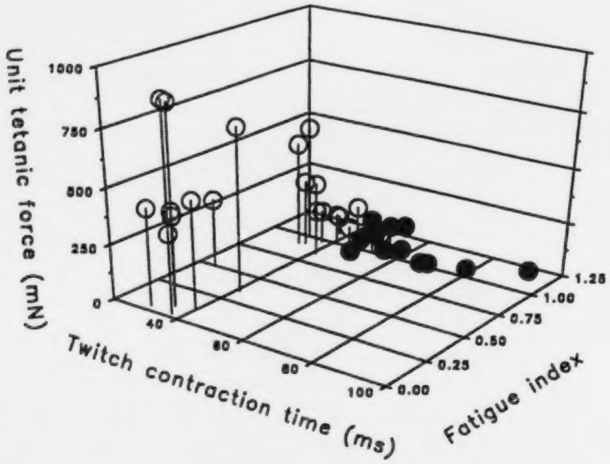
5.3 1 MU classification

Fig. 5.1 shows multivariate physiological profiles of single MG units based on contraction time, fatigue index, and tetanic force, displayed on 3-dimensional graphs. Normally, MUs in the cat MG muscle can be separated into fast-contracting (F units) and slow-contracting (S units) units according to the presence (F units) or absence (S units) of sag during an unfused tetanus (Burke et al., 1973; Burke, 1981). S units also typically have relatively long contraction times (>40 ms), produce small force values, and do not fatigue during a 2 minute fatigue test (Burke et al. 1973) (Filled symbols; Fig. 5.1A). F units have shorter contraction times (<40 ms), and produce relatively large tetanic forces (open symbols; Fig. 5.1A). F units can be further subclassified into FR, FI and FF units according to their resistance to fatigue during intermittent tetanic stimulation at 25 Hz (Burke fatigue test; Burke et al. 1973). FR units are fatigue resistant with a fatigue index greater than 0.75, while FF units are fatigable with a fatigue index less than 0.25. A small number of normal fast cat MG units have fatigue index values between 0.25 and 0.75 and are referred to as FI units. Despite a continuous distribution of twitch-contraction times and tetanic force values for all MUs there is very little overlap in these contractile properties between F and S units such that S units contract more slowly and are generally weaker than the larger F units. In addition, F units exhibit a clear bimodal distribution of fatigue indices which easily separates FF from FR units (see also Burke et al. 1973). Therefore, using the

Figure 5.1.

MG motor units in 1 normal (A) and 3 partially denervated muscles (B) innervated by ~20% (mean ± S.E.: 19.5 ± 2.7%) of their normal complement of MUs plotted as a function of twitch contraction time, fatigue index, and tetanic force on 3-dimensional graphs. Filled circles denote MUs that did not sag during an unfused tetani (type S units). Despite a continuous distribution of unit twitch contraction times and tetanic force, S units in normal muscles show little overlap with F units with respect to these contractile properties. The tetanic force of all MUs in extensively partially denervated muscles increased their force generating capacity (note Z-axis in B is 4 times that in A). The distribution of MUs is similar in partially denervated muscles however a few large MUs show atypical "no sag" characteristics with fast contraction times and/or low fatigue indices.

A. Normal


Unit tetanic force (mN)
1000
750
500
250
0
Twitch contraction time (ms)
40
60
80
100
Fatigue index
0.00
0.25
0.50
0.75
1.00
1.25

B. PD

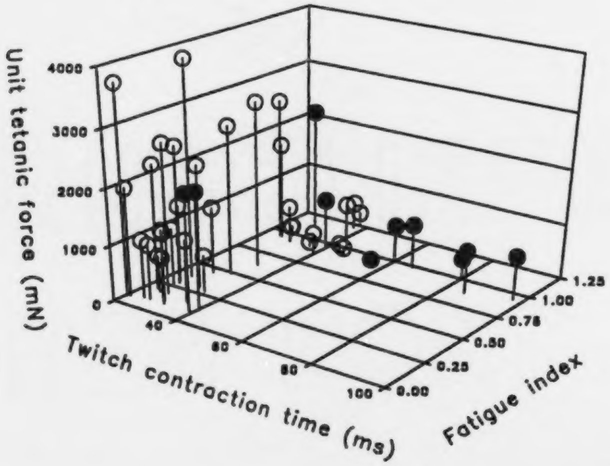

Unit tetanic force (mN)
4000
3000
2000
1000
0
Twitch contraction time (ms)
40
60
80
100
Fatigue index
0.00
0.25
0.50
0.75
1.00
1.25

Table 5.1. Percent MU types

Type	S	FR	FI	FF	UC
Normal	25 ± 2.3	26 ± 2.0	8 ± 2.0	42 ± 3.0	-
PD	19 ± 7.3	23 ± 4.6	16 ± 3.6	29 ± 3.8	12 ± 2.8
Crush	14 ± 2.5	9 ± 4.4	16 ± 3.8	33 ± 5.7	24 ± 4.6
N-N	20 ± 4.2	14 ± 4.4	28 ± 6.8	26 ± 5.9	16 ± 7.4
N-M	14 ± 4.9	21 ± 5.2	27 ± 7.3	13 ± 4.7	25 ± 9.6

Values are means ± S.E. PD, partially denervated muscles; Crush, reinnervated muscles after nerve crush; N-N, muscles reinnervated after N-N suture; N-M, muscle reinnervated after N-M suture. S, Slow units; FR, fast-fatigue resistance; FI, fast-fatigue intermediate; FF, fast-fatigable; UC, unclassifiable motor units.

classification criterium of sag, contraction time, and fatigue index, normal cat MG units can be separated into 4 groups with tetanic force increasing in the order of $S \leq FR < FI = FF$ as shown in Fig. 5.1A.

The majority of MUs sampled in partially denervated muscles can be classified as S or F units according to the criteria of sag (Table 5.1; Fig. 5.1B). As shown in Fig. 5.1B, the MU profiles were very similar to normal despite the fact that, in this example, only 25% (19.5 ± 2.7%, n=3) of the normal complement of MUs remained in the muscle after sectioning the ventral root.

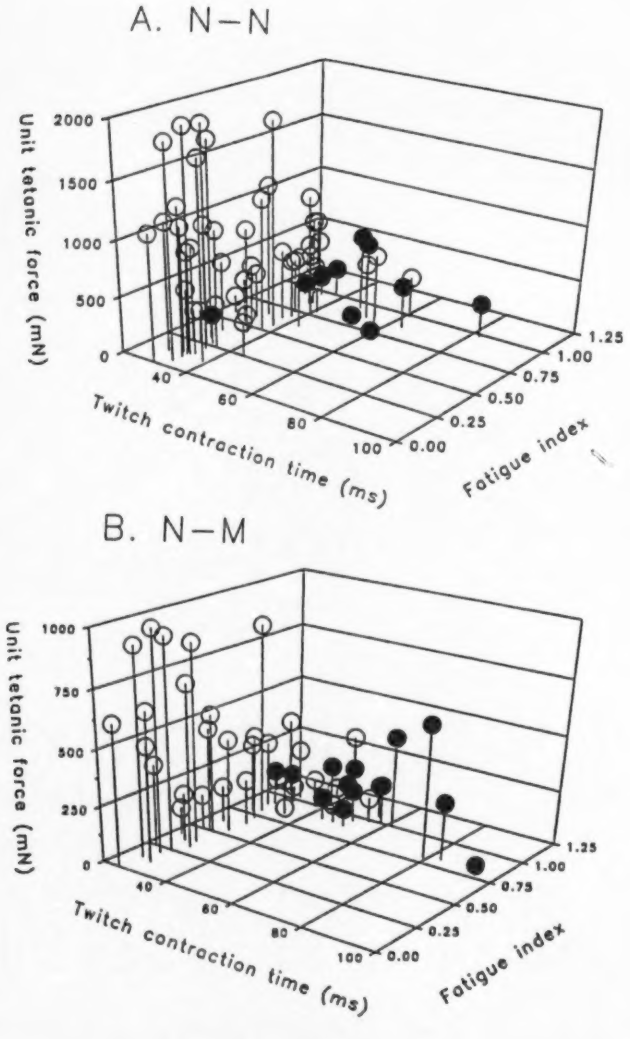
However, in partially denervated muscles sag was less reliable in separating S from F units as indicated by 4 MUs that did not sag but had contraction times <40 ms. Two of the MUs were also fatigable (fatigue index <0.25); a trait atypical to normal "no sag" MUs. These reinnervated MUs therefore differ from normal and have been labelled, for the purpose of this paper, as unclassifiable (UC) MUs (Table 5.1). In addition, there was also an increase in the number of FI units in partially denervated muscles (Table 5.1).

The tetanic force of MUs increased significantly in partially denervated muscles when only 25% of their normal complement of MUs remained intact (Fig. 5.1B; also described in detail in Chapter 2). Note that the force axis in Fig. 5.2B is 4 times that in Fig. 5.2A. All MUs increased by the same factor such that the normal ordering of MUs according to force was maintained (ie. $S \leq FR < FI = FF$).

As was observed in partially denervated muscles (Fig. 5.1B), sag was less reliable for separating S from F units in muscles reinnervated by <50% of their MUs

Figure 5.2.

MUs sampled from reinnervated muscles after complete MG nerve transection and repair with either N-N (A) or N-M sutures (B) plotted as in Fig. 5.1. MUs were sampled from 2 muscles reinnervated by 36 ± 2.8% (A) and 41 ± 6.7% (B) of their normal complement of MUs. Unit force increased in reinnervated muscles after N-N, but not after N-M suture (note Z-axis is 2 times greater in A than B). Generally, the distribution of MUs in reinnervated muscles are similar to normal with the exception of an increase in FI units (fatigue index >0.25 and <0.75) and a few atypical MUs that did not sag but had relatively fast contraction times (<40 ms) or low fatigue indices (<0.75).


A. N-N
Unit tetanic force (mN)
2000
1500
1000
500
0
40
60
80
100
Twitch contraction time (ms)
1.25
1.00
0.75
0.50
0.25
0.00
Fatigue index
B. N-M
Unit tetanic force (mN)
1000
750
500
250
0
40
60
80
100
Twitch contraction time (ms)
1.25
1.00
0.75
0.50
0.25
0.00
Fatigue index

after N-N (Fig. 5.2A) or N-M suture (Fig. 5.2B). That is, some MUs did not exhibit a sag response but were fast contracting and\or fatigable. However, the most obvious difference in the reinnervated MU profiles was the large increase in FI units (Table 5.1). Unit tetanic force also increased in muscles reinnervated after N-N, but not N-M suture as demonstrated by the presence of MUs with force values >1000 mN in Fig. 5.2A, but not Fig. 5.2B. In addition, MUs of different types were more similar in their tetanic force output such that the normal order of MUs according to force (ie. $S \leq FR < FI \leq FF$) is less evident. This result was most striking in muscles reinnervated after N-M suture (see below).

Fig. 5.3 shows that distribution of unit twitch contraction times in normal cat MG muscles (Fig. 5.3A), partially denervated muscles (PD, Fig. 5.3B), and reinnervated muscles after MG nerve crush (Fig. 5.3C), or after nerve transection and resuture with N-N (Fig. 5.3D) or N-M sutures (Fig. 5.3E). Normally, the cat MG muscle contains a higher proportion of F compared to S units as evident by the skewed distribution to faster contracting MUs. The means, ranges and distributions of unit twitch contraction times in partially denervated, and reinnervated muscles were very similar to normal. Thus, enlarged MUs in partially denervated muscles, and in reinnervated muscles, have the normal range of contractile speed and proportions of F and S units. Similarly, the mean and range in the half fall time of twitch contractions for MUs in partially denervated (Fig. 5.4B) and reinnervated muscles after nerve crush (Fig. 5.4C), N-N (Fig. 5.4D), and N-M suture (Fig. 5.4E) were not different from normal (Fig. 5.4A).

Fatigability, in contrast to contractile speed, was not the same as normal for all

Figure 5.3.

Frequency histograms of unit twitch contraction times (ms) in normal (A), partially denervated (B) and reinnervated muscles after nerve crush (C), nerve transection with N-N (D), or N-M sutures (E). Mean (± S.E.) number (%) of MUs in each experimental condition are: 40 ± 7.5% (n=10, B), 44 ± 9.1% (n=7, C), 49 ± 8.1% (n=9, D), and 45.7 ± 8.8% (n=7, E). The mean and range in unit twitch contraction times are similar to normal in B-E. Mean (± S.E.) are shown by vertical lines and are: 42.2 ± 1.2 (A), 38.2 1.2 (B), 41.0 ± 1.2 (C), 38.9 ± 1.1 (D), and 44.3 ± 1.6 (D).

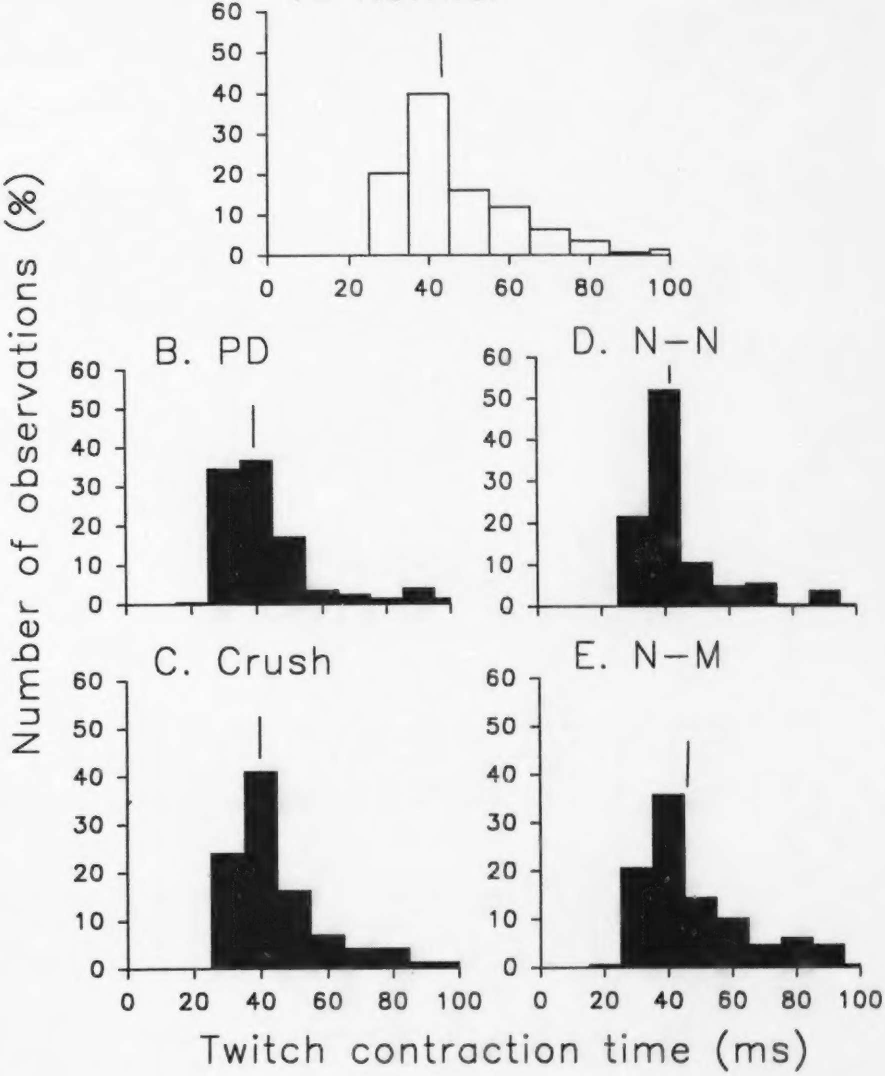

A. Normal
B. PD
C. Crush
D. N–N
E. N–M
Number of observations (%)
Twitch contraction time (ms)
0
10
20
30
40
50
60
0
20
40
60
80
100

Figure 5.4.

Frequency histograms of unit half fall times in normal (A), partially denervated (B), and reinnervated muscles after nerve crush (C), N-N (D), or N-M sutures (E). MUs were sampled from the same muscles as in Fig. 5.3. Mean (± S.E.) are shown by vertical lines and are: 30.2 ± 2.1 (A), 36.8 ± 3.4 (B), 41.6 ± 5.3 (C), 35.6 ± 1.5 (D), and 39.0 ± 1.9 (E).

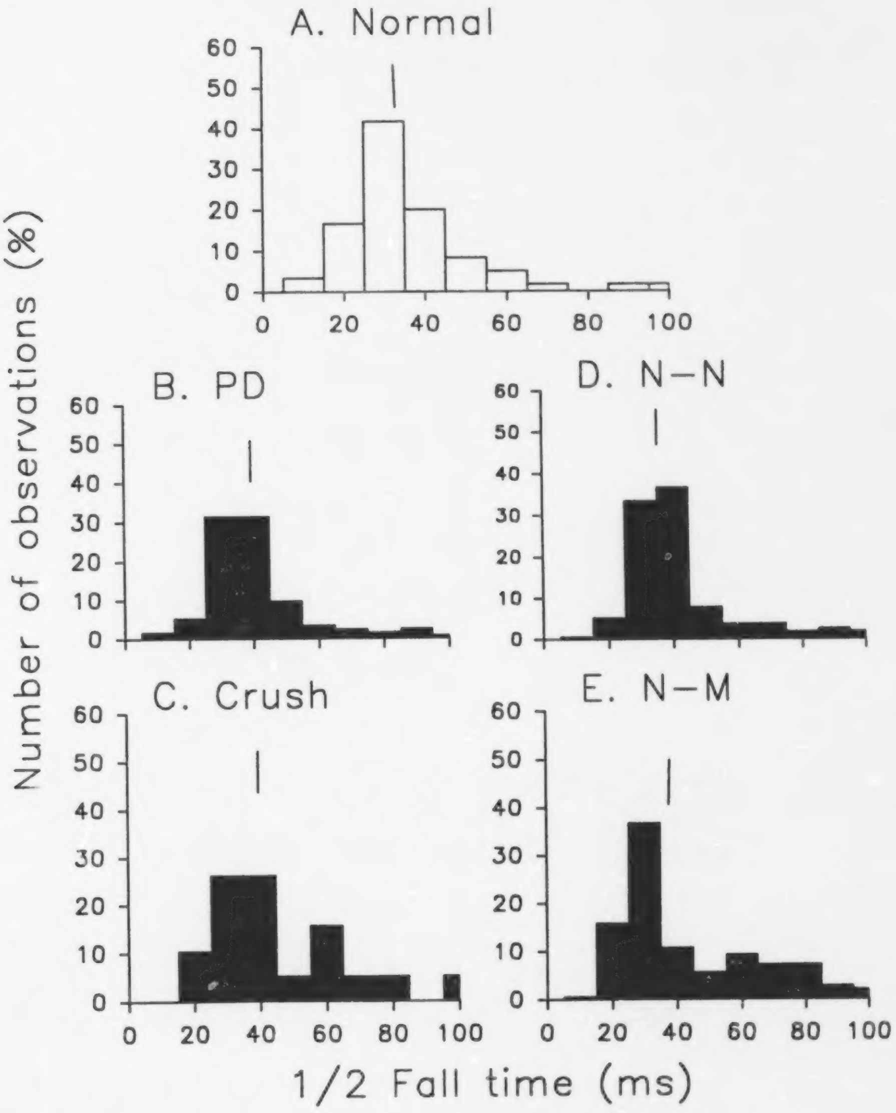
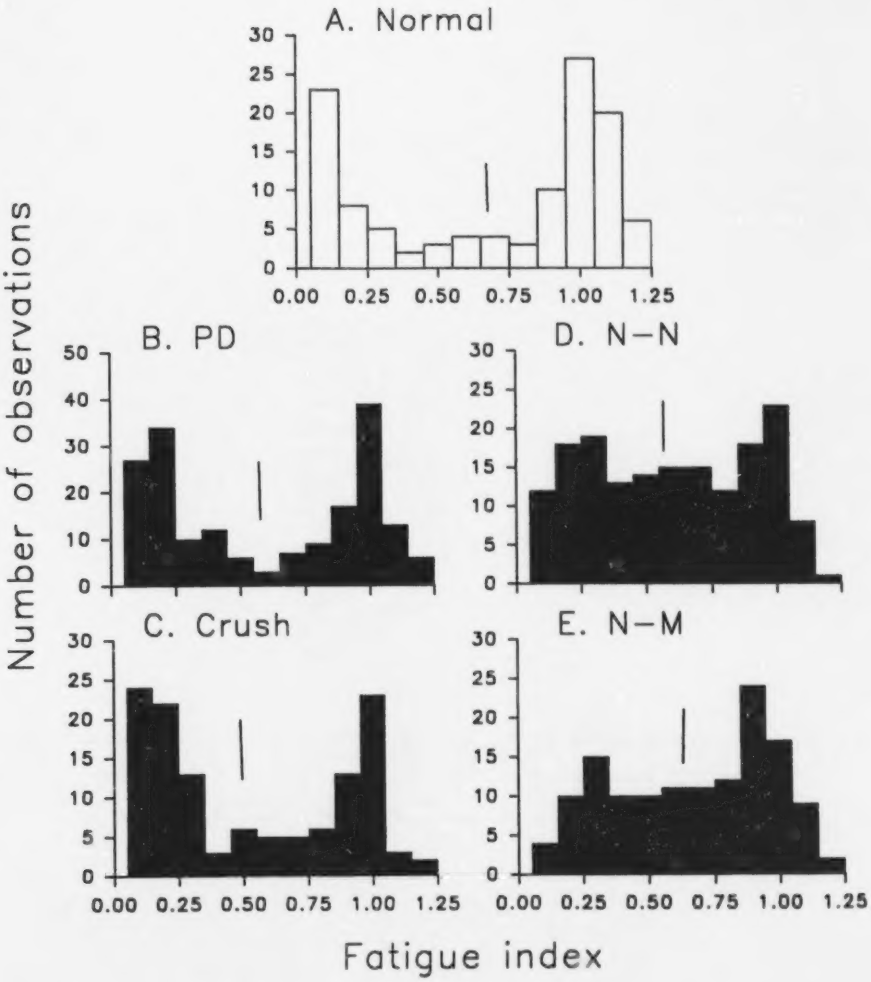

A. Normal
B. PD
C. Crush
D. N–N
E. N–M
Number of observations (%)
1/2 Fall time (ms)
0
10
20
30
40
50
60
0
20
40
60
80
100

Figure 5.5.

Distribution of unit fatigue index sampled from normal (A), partially denervated (B) and reinnervated muscles after nerve crush (C), N-N (D), or N-M sutures (F). The distribution of fatigue index values are bimodally distributed in normal, partially denervated and reinnervated muscles after nerve crush, but is less discretely divided into 2 modes in muscles reinnervated by completely transected nerves (ie. N-N and N-M sutures).


A. Normal
B. PD
C. Crush
D. N-N
E. N-M
Number of observations
Fatigue index
0
5
10
15
20
25
30
40
50
0.00
0.25
0.50
0.75
1.00
1.25

conditions as shown in Fig. 5.5. Normally, fatigue index values are bimodally distributed (Fig. 5.5A) reflecting the similar proportions of nonfatigable (S and FR) and fatigable MUs (FF) in the cat MG muscle with relatively few MUs having fatigue indices between 0.25 and 0.75 (FI units). When motor axons innervate at least some of their original muscle fibers, as is the case in partially denervated and reinnervated muscles after nerve crush, the bimodal distribution is reestablished (Fig. 5B,C) and there is only a moderate increase in the number of FI units (Table 5.1). When nerves do not reinnervate their original muscle fibers, as is the case following complete nerve transection (ie. N-N and N-M suture), there is a substantial increase in the proportion of FI units and the distribution approaches unimodality (Fig. 5.4D,E).

In summary, when the number of intact and regenerating nerves are experimentally reduced and MUs are forced to increase in size to compensate for the smaller MU number, the majority of MUs can still be classified as S, FR, FI, and FF. These results support the view that MU characteristics are, at least in part, regulated by the reinnervating nerve. However, the presence of UC units as well as the increased number of MUs with intermediate fatigue characteristics suggests that there is incomplete conversion of muscle fiber properties by the reinnervating motor nerve (see Discussion).

5.3.2 MU contractile force and size relationships

Although mean unit force normally increases in order of type (S<FR<FI=FF) there is substantial overlap in the force distributions (Fig. 5.6, open histograms).

However, note that there is little overlap between the S and FI,FF units. Following partial denervation (Fig. 5.6, filled histograms) or reinnervation after nerve crush (not shown), when the MU number is 25% that of normal, the tetanic force of all 4 MU types increased by a similar extent such that the normal range and order of increasing force between MU types were maintained (ie. $S \leq FR < FI = FF$). As observed in normal muscles, there was very little overlap between the force values of the S and FF units.

Unit force is the product of IR, mean muscle fiber CSA and the specific force (SF) or force per unit area (Burke 1981). Therefore an increase in unit force in partially denervated muscles may be due to an increase in 1 or all 3 of these factors. As discussed in detail in Chapter 2, the observed increase in unit force in partially denervated muscles is not due to hypertrophy of muscle fibers but is primarily due to an increase in IR. As illustrated in Fig. 5.7, for a partially denervated (PD) muscle, innervated by only 25% of its normal complement of MUs, the mean and range of all three fiber types becomes very similar (see also Fig. 2.8). As a result, the range in unit force, and differences in force between MU types, cannot be attributed to differences in fiber size. Comparison of the mean and range of fiber size in reinnervated muscles after partial denervation, crush, or nerve sections, shows the very striking trend for all fibers to become similar in size, primarily because the range of FG fibers is dramatically reduced (see Chapter 3).

The normal size relationships between axon potential amplitude and unit tetanic force returned after partial denervation or crush injuries as shown in the examples in Fig. 5.8 (A,C,E, respectively) where $\sim$25% of the normal complement of MG MUs

Figure 5.6.

Distribution of tetanic force developed by each of FF, FI, FR and S units in 2 normal and 2 partially denervated muscles innervated by ~20% (X ± S.E.: 19.8 ± 3.7%) of its normal complement of MUs. The force of all 4 MU types increased significantly in partially denervated muscles, but the range remained the same. Mean values are indicated by the vertical line to show that $S \leq FR < FI = FF$ in normal and partially denervated muscles.

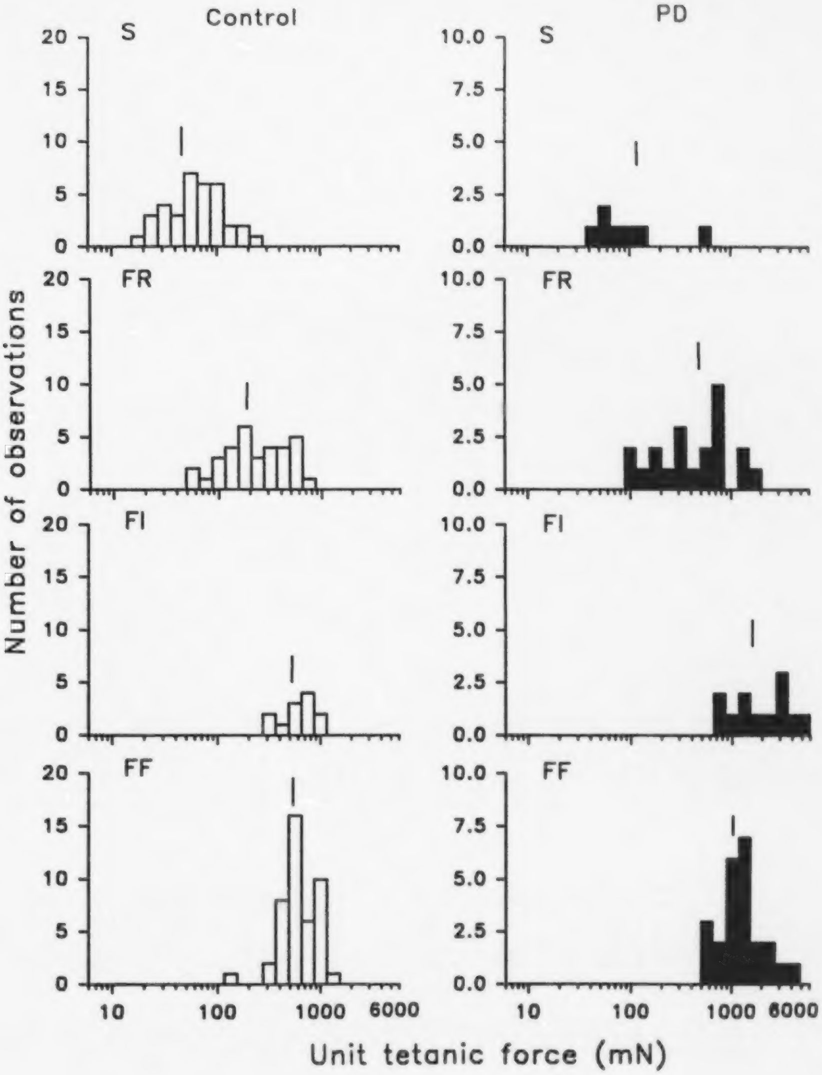
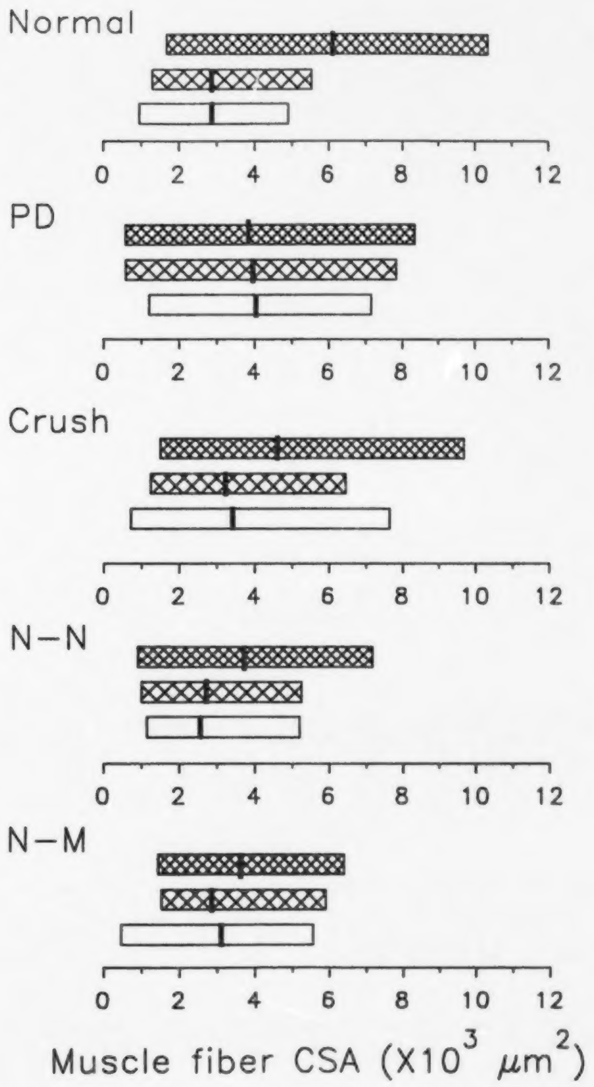

Control
PD
S
FR
FI
FF
Number of observations
0
5
10
15
20
0.0
2.5
5.0
7.5
10.0
10
100
1000
6000
Unit tetanic force (mN)

Figure 5.7.

Ranges in CSA of each muscle fiber type in normal, partially denervated (PD), and reinnervated muscles after nerve crush, nerve transection with N-N, or N-M sutures. All experimental muscles were reinnervated by ~25% of their normal complement of MUs. FG (dense cross-hatched), FOG (cross-hatched), and SO fibers (empty bars).


Normal
PD
Crush
N–N
N–M
0
2
4
6
8
10
12
Muscle fiber CSA (X10^3 μm^2)

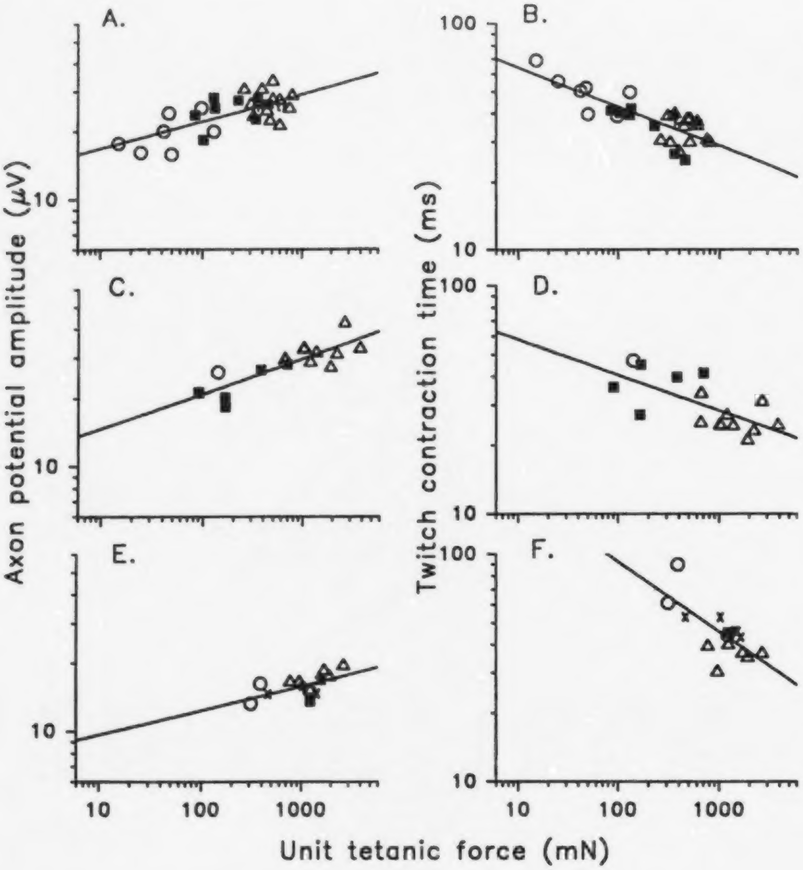
reinnervated the muscles. The slopes (± S.E.) of the regression lines were very similar being: 0.12 ± 0.02 (RO: 0.60); 0.15 ± 0.03 (RO: 0.83); 0.19 ± 0.06 (RO: 0.53) for normal, partially denervated, and reinnervated muscles after nerve crush, respectively. The slopes are significantly different from zero, and not different from each other ($p<0.05$). Since the range in unit force cannot be attributed to differences in muscle fiber CSAs (Fig. 5.7) these results indicate that the number of muscle fibers supplied by normal and reinnervated motoneurons in partially and completely denervated muscles after nerve crush, respectively, is directly related to axon potential amplitude, and therefore, axon size.

The normal inverse relationship between twitch contraction time and unit tetanic force was also reestablished in these muscles (Fig. 5.8B,D,F). The slopes (± S.E.) of the regression lines were: -0.14 ± 0.03 (RO: 0.66); -0.16 ± 0.05 (RO: 0.68); -0.28 ± 0.07 (RO: 0.70) for normal, partially denervated, and reinnervated muscles after nerve crush, respectively. All slopes are significantly different from zero ($p<0.05$). Following nerve crush, the size of the reinnervated S unit increased proportionately more than the FF units (open triangles) as shown by the larger shift of the S (filled circles) units along the X-axis in Fig. 5.8E (see also Fig. 3.5). This greater increase in size of the S units accounts for the steeper relationship between twitch contraction and unit tetanic force in this muscle reinnervated after nerve crush as compared to normal (cf. Fig. 5.8B,F).

Unlike the proportional increase in tetanic force of all MU types in partially denervated muscles (Fig. 5.6), force of S units increased their force generating capacity

Figure 5.8.

Axon potential amplitude and twitch contraction time plotted as a function of tetanic force in MUs from normal (A,B), partially denervated muscle innervated by 25% of its MUs (C,D), and muscles reinnervated after nerve crush by 25% of its MUs (E,F). S units indicated by open circles, FR filled boxes, FI and FF by open triangles and UC units by crosses. The slopes (± S.E.) of the regression lines for axon potential amplitude and tetanic force (0.12 ± 0.02, 0.15 ± 0.03, 0.19 ± 0.06, RO= 0.60, 0.83, 0.53, respectively) are all significantly different from zero ($p<0.01$-0.05) and not different from each other. The negative slopes of the regression lines for contraction time and tetanic force (0.14 ± 0.02, 0.16 ± 0.05, 0.28 ± 0.07; RO= 0.66, 0.68, 0.70, respectively) are also significantly different from zero. Slopes of B,D are not different from each other, however slope in F is significantly steeper.


A.
B.
C.
D.
E.
F.
Axon potential amplitude (μV)
Twitch contraction time (ms)
Unit tetanic force (mN)
10
100
1000

to a greater extent than the F units in reinnervated muscles after nerve section and repair (Fig. 5.9; see also Fig. 3.5). As a result, there was more overlap in the range of unit force between MU types and the overall range in unit force decreased (Fig. 5.9). This trend was particularly noticeable in muscles reinnervated after N-M suture where FI and FF forced increased very little due to the restricted growth environment (Chapter 4).

Despite the overlap in the force output of the different MU types, the correlation between axon potential amplitude and unit tetanic force was observed in reinnervated muscles when the number of reinnervated MUs was greater than 50% of normal, irrespective of the method of nerve repair (ie. N-N or N-M) (Fig. 5.10A,C). If 50% or less of the normal complement of MUs reinnervate the muscle the normal size relationship between axon potential amplitude and unit force was reestablished after N-N (Fig. 5.10C), but not after N-M suture (Fig. 5.10D) as shown for two examples in Fig. 5.10. This loss of the normal size relationship may be explained by 1) the reduced FG fiber size which decreased FF unit force significantly so that unit force underestimates the increase in IR and 2) S units increased in size to a greater extent than FR and FF units (see Discussion for details).

5.3.3 CSA of muscle unit fibers from single MUs

The range of muscle fiber CSA is significantly less for SO as compared to FG fibers as shown in Fig. 5.11A (filled boxes). Even within single MUs (open boxes), muscle fibers differ considerably in size. This is particularly striking for FG fibers in FF units (Fig. 5.11A, open boxes). For FF units, where tetanic forces ranging from

Figure 5.9.

Distribution of tetanic force developed by each of FF, FI, FR and S units in muscles reinnervated by ~25% of their normal complement of MUs after complete nerve transection and repair with N-N or N-M. The force of S units increased significantly in partially denervated muscles, but the range remained the same. Mean values are indicated by the vertical line to show that $S < FR < FI \leq FF$ in normal and in reinnervated muscles after N-N and N-M sutures.

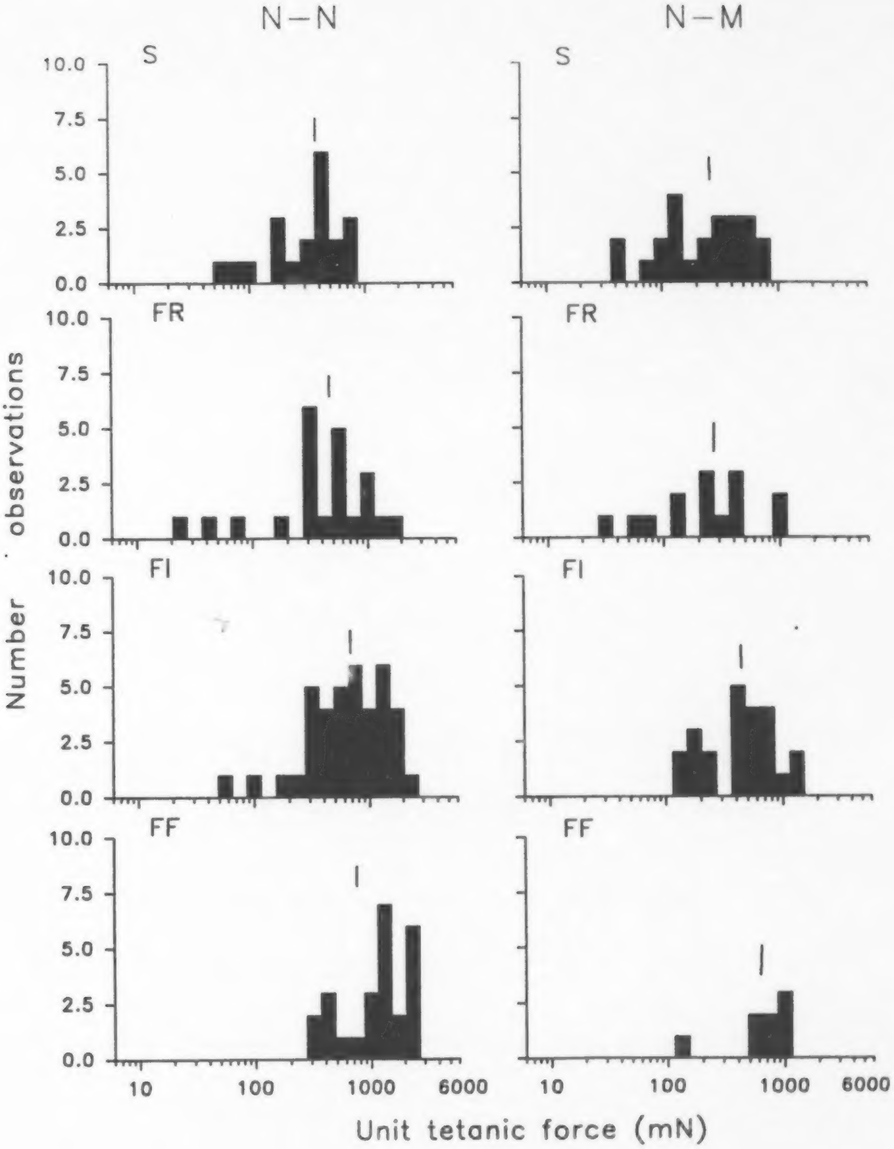
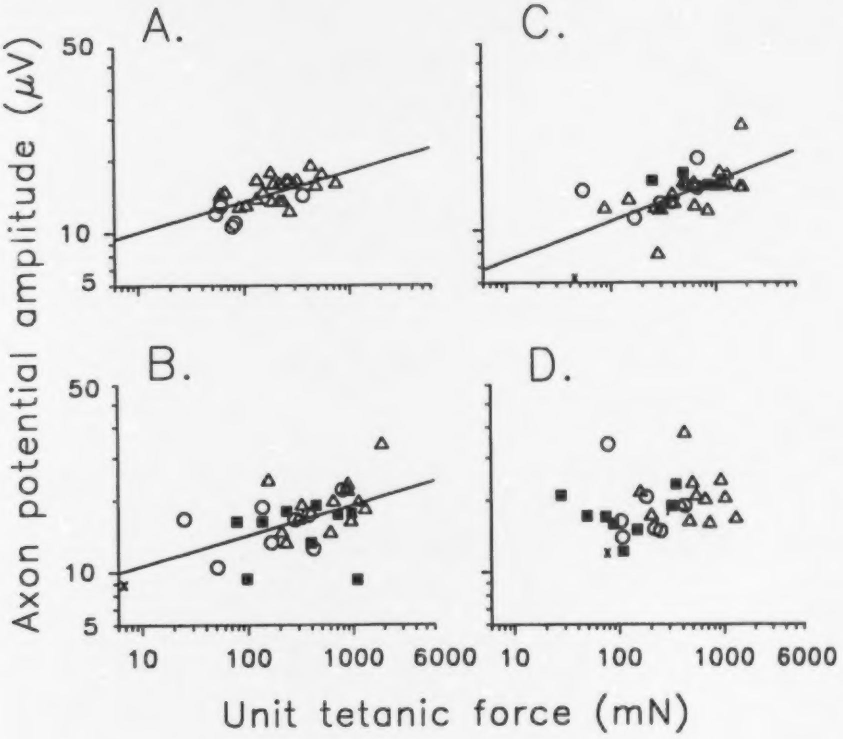

N–N
N–M
S
FR
FI
FF
10.0
7.5
5.0
2.5
0.0
Number of observations
10
100
1000
6000
Unit tetanic force (mN)

Figure 5.10.

Axon potential amplitude plotted as a function of tetanic force in MUs from muscles reinnervated by >50% (A,C) and <50% (B,D) of their normal complement of MUs after N-N (A,B) or N-M sutures (C,D). S units indicated by open circles, FR filled boxes, FI and FF by open triangles. Muscles in A,B,C,D were reinnervated by 88%, 32%, 88%, and 31% of their MUs, respectively. The slopes ($\pm$ S.E.) of the regression lines in A-C (0.13 $\pm$ 0.03, 0.13 $\pm$ 0.03, 0.12 $\pm$ 0.02; RO= 0.63, 0.58, 0.67, respectively) are all significantly different from zero ($p<0.01$-0.05) and not different from each other or from normal (Fig. 5.7A). The regression line fitted to the values in D is not significantly different from zero ($p<0.05$) and therefore has not been drawn.

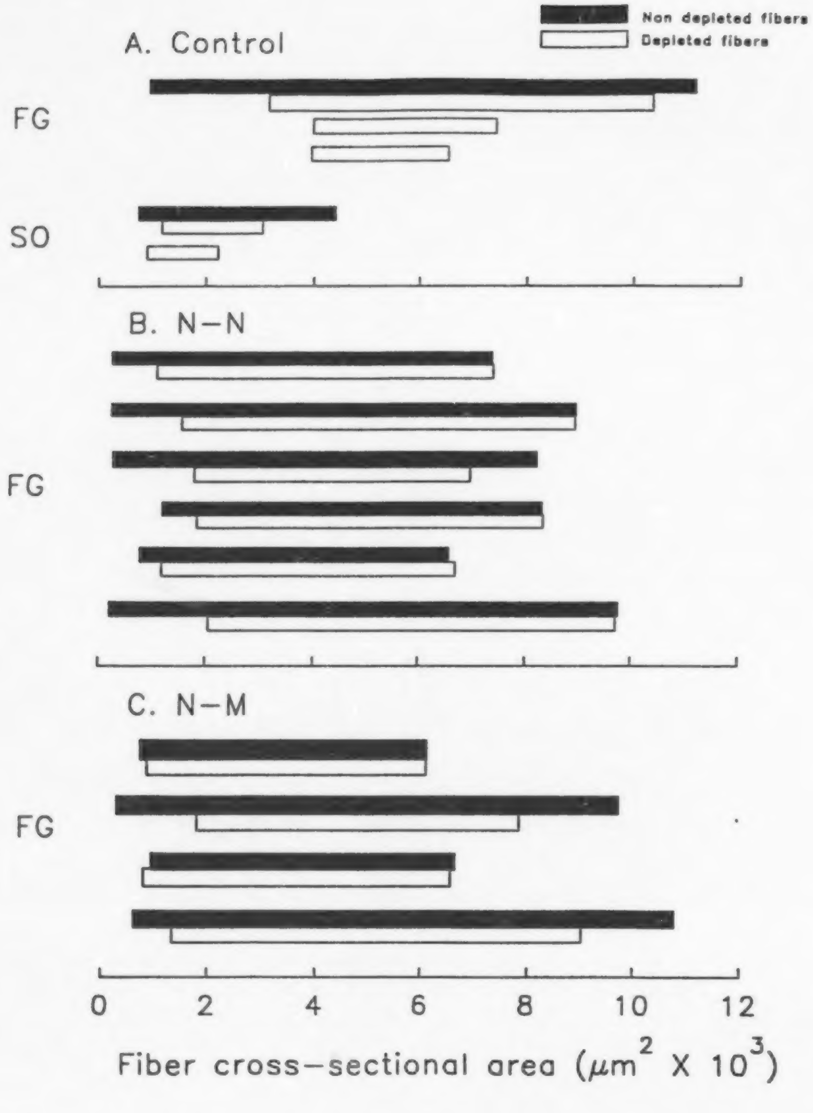

A.
B.
C.
D.
50
10
5
Axon potential amplitude (μV)
10
100
1000
6000
Unit tetanic force (mN)

125-528 mN, muscle fiber CSA varied by 2.5 to 7.5- fold. The range in muscle fiber CSA within a single FF unit was always less than that found within a population of FG fibers belonging to other MUs of the same type (ie. non-depleted FG fibers) (compare open and filled boxes).

After reinnervation, the range in fiber CSA within a single FF unit was very similar to that found within a population of FG fibers sampled from the muscle. The overlap in ranges of fiber size for 10 glycogen-depleted reinnervated MUs (open boxes) and the samples of nondepleted fibers (cross-hatched boxes) is schematized in Fig. 5.11B,C. This tremendous overlap in the range between depleted and nondepleted muscle fibers suggests that there is an increase in heterogeneity in fiber size within a single MU as compared to normal.

Figure 5.11.

Ranges in fiber CSA from depleted muscle unit fibers (empty bars) and non-depleted fibers of the same histochemical types (filled bars) for 5 normal (3 FF units; 2 S units) and 10 reinnervated MUs (6 N-N; 4 N-M). FG, fast-glycolytic; SO, slow oxidative.


Non depleted fibers
Depleted fibers
A. Control
FG
SO
B. N–N
FG
C. N–M
FG
0
2
4
6
8
10
12
Fiber cross–sectional area (μm^2 X 10^3)

5.4 DISCUSSION

The results of these studies where few motor nerves were forced to innervate more muscle fibers than normal shows 1) neural control of muscle fiber properties may be less than anticipated from earlier studies and 2) that size dependent branching of sprouting and regenerating nerves is the primary factor that restores the range in unit force in reinnervated muscles.

5.4.1 Evidence for heterogeneity in reinnervated MUs

Normally MUs are classified as S, FR, FI, and FF based on 1) the presence or absence of sag, respectively, during an unfused tetani, 2) twitch contraction time and 3) fatigue characteristics, as originally described by Burke et al. (1973). All 4 MU types were also found in muscles after either partial denervation, reinnervation after nerve crush, or after complete nerve transection and repair with N-N or N-M sutures, however there were significantly more FI units in experimental muscles as compared to normal (Table 5.1).

Between 12 to 25% of the MUs sampled in the experimental muscles were unclassifiable (UC; Table 5.1) using sag and fatigue criteria because they either fatigued, but did not elicit a sag response or, they did not sag, yet had fast contraction times (<35 ms) and/or were fatigable. These MUs therefore appear to have properties intermediate to F and S units characterized in normal MG muscles. The motoneuron therefore does not appear to exclusively determine contractile properties of reinnervated muscle fibers (see also Chan et al. 1982; Dum et al. 1985b; Foehring et al. 1987b; Gordon et al. 1988; Tötösy de Zepetnek et al 1992).

The occurrence of sag has been described as a subtle change in the fiber active state (Burke et al. 1973) which appears to decrease the period of effective force generation of the muscle fibers during the time course of the unfused tetani (Burke et al. 1976). The different sag response elicited by F and S units may be due to differences in the sarcoplasmic reticulum (SR) between the two MU types (Burke et al. 1976). The "inappropriate" sag responses observed by some MUs may possibly represent incomplete conversion of SR properties by the innervating motoneuron (see below).

5.4.2 Contractile speed

At the MU level, and muscle fiber level, reinnervated MUs can be classified by their physiological and histochemical parameters of contractile speed, and mATPase. MUs of each type showed the normal range of speed and the fibers in each MU stained uniformly with acid or alkali ATPase. Thus, even under conditions where each nerve supplies many muscle fibers that initially belonged to several different MUs, the normal contractile speed and histochemical types are reestablished supporting the view that MU contractile and histochemical properties are regulated by the innervating motoneuron. However, the range in twitch contraction time within a single MU type, even under normal conditions, can vary over a 2- fold range (Fig. 5.1) without any gradation in mATPase staining (Burke 1981). This normal variance in contractile speed of the same fiber type can be attributed to a continuum of myosin heavy chain composition within single muscle fibers which is not detected by histochemistry as well as possible differences in the rate of Ca^{2+} release and uptake into the SR (Pette and Staron 1990). Where there is a range, the volume density of the terminal cisternae of the SR of muscle fibers is inversely related to twitch contraction (Kugelberg and Thornell 1983).

There are examples, after reinnervation, particularly for reinnervated soleus muscles, where neural transformation of muscle fiber properties is incomplete and muscle fibers retain their original phenotype (Chan et al. 1982; Dum et al. 1985b; Foehring et al 1987a). Contraction times of cat soleus MUs, cross-reinnervated with motor axons to the MG and FHL muscles, are significantly slower than normal MG and FHL units (Chan et al. 1982; Foehring et al. 1987a). In addition, the significant increase in F units in reinnervated rat soleus muscles was not matched by an increase in FG fibers (Gillespie et al. 1986). Finally, immunohistochemical analysis of reinnervated muscle fibers have shown that both fast and slow myosin heavy and light chains are coexpressed in the same muscle fiber (Gauthier et al 1983). In support of this heterogeneity in muscle fiber composition following reinnervation, recent findings in our lab have shown that there is an increase heterogeneity in myosin heavy chains within a single MU in reinnervated rat TA muscles (Fu et al. 1992).

5.4.3 Fatigability

There was a dramatic increase in the number of FI units in enlarged reinnervated MUs following reinnervation after complete nerve transection (N-N or N-M suture) (Table 5.1, Fig. 5.5) suggesting that MUs may be heterogeneous in their fatigue properties possibly due to incomplete conversion of metabolic enzymes in reinnervated muscle fibers (Gordon et al. 1986;1988). More FI units have been previously noted in reinnervated muscles (Gordon et al. 1986;1988), but the difference in the number of FI units under different reinnervation conditions argues well for incomplete conversion of fatigue properties by the nerve.

Interestingly, there was only a moderate increase in FI units in partially denervated muscles and completely denervated muscles after nerve crush (Table 5.1, Fig. 5.5). The smaller number of FI units in these muscles can be explained, at least in part, by the fact that intact nerves and regenerating nerves after nerve crush innervate their original muscle fibers. In contrast, where nerves do not reinnervate any, or very few, of their original muscle fibers after nerve section and repair, the number of FI units increased dramatically (Fig. 5.2). However, since the size of MUs in partially denervated muscles, and reinnervated muscles after nerve crush, increased in size by as much as 4-6 times normal when the number of MUs was dramatically reduced (see Chapter 3), the motor axon reinnervated a large proportion of fibers previously from different MUs. During the fatigue test, it may be that the most fatigue resistant muscle fibers maintain unit force so that the fatigue profile is a reflection of the average fatigability. Differences in MU fatigabilty may, therefore, only be discernable if the duration of the fatigue test is extended.

The increase occurrence of FI units in reinnervated muscles may be due to coexistence of muscle fibers within the same MU that express glycolytic or oxidative enzymes (Gordon and Pattullo 1992). Fatigue resistance is normally positively correlated with oxidative capacity as indicated by the level of succinate dehydrogenase (SDH) activity in isolated MUs that vary in fatigability (Kugelberg and Lindegren, 1979). Consistent with incomplete conversion of muscle fiber metabolic enzymes by the reinnervating motor axon the level of SDH activity is more divergent between fibers of a single reinnervated MU as compared to normal MUs (Rafuse et al. 1988; Unguez et al. 1992).

5.4.4 Muscle fiber CSA

The range in muscle fiber size, even in normal MUs, varies over a 2.5 to 7.5-fold range (Fig. 5.11A). This range is similar to the 2 to 8- fold range found within cat TA units (Bodine et al. 1987) and 2 to 6-fold range in rat TA muscles (Tötösy de Zepetnek et al. 1992a). Since it may be assumed that all muscle fibers within a single MU are subjected to the same amount of neural influence, this heterogeneity indicates that fiber size is not entirely neurally regulated (see also Edstrom and Kugelberg 1968; Tötösy de Zepetnek et al. 1992a). A recent study by Edgerton and colleagues (Ounyian et al. 1991) shows that the CSA of a single cat TA muscle fiber varies significantly along its length. In addition, muscle fibers of the same type vary in size from the deep to superficial regions such that the largest fibers tend to be located superficially (see also Pullen 1977). This gradation in size may reflect variations in mechanical loading of muscle fibers in different regions of the muscle (Gordon and Pattullo 1992). This is supported by the observation that muscle fibers increased in size in chronically stimulated immobilized muscles under conditions where the contractions were mainly isometric, but decreased in size if the same pattern of stimulation was applied to the muscle immobilized in a shortened position (Cotter and Phillips 1986).

Following reinnervation, the range in muscle fiber size within a single glycogen-depleted FF unit was very similar to the range in size of all FG fibers measured in the whole muscle cross-section (Fig. 5.11B,C). In addition, there was extensively overlap in size between fibers of different types in reinnervated muscles after N-N or N-M suture (Fig. 5.7). The heterogeneity in the size of muscle fibers within a single MU, following reinnervation, clearly indicates that fiber size is not solely determined by the motoneuron (Edstrom and Kugelberg 1968; Tötösy de Zepetnek et al. 1992). This increased range in size may reflect differences in the mechanical loading of the muscle fibers during contraction or may be due to intrinsic differences in the muscle fibers that are established during development and are not under neural control (Butler et al. 1982; Dhoot 1985; Miller and Stockdale 1987).

Taken together, these data show that neural control of muscle fiber properties may be less than anticipated from the results of Buller et al. (1960). Either mechanical loading or intrinsic developmental factors of muscle fibers may set the range in which muscle fiber properties can be modulated by the innervating motoneuron.

5.4.5 Size relationships

The normal size relationship between axon potential amplitude and unit tetanic force was reestablished in partially denervated muscles and reinnervated muscles when the majority of the MUs (>50%) innervated the muscle. Size relationships also returned in partially denervated and reinnervated muscles after MG nerve crush or complete nerve transection and repair with N-N sutures when the number of MUs was severely reduced (<50%) (Figs. 5.8,5.10). No correlation between axon size and unit tetanic force occurred in muscles after N-M suture when <50% of the normal complement of MUs reinnervated the muscle (Fig. 5.10).

Since extracellularly recorded axon potential amplitude is an electrophysiological measure of axon size (Gordon and Stein 1982a) and differences in fiber CSA between different types in normal and reinnervated muscles cannot account for the 100- fold range in MG unit tetanic force these results show that the size relationship is primarily due to size dependent branching of the motor axon in normal and reinnervated muscles. These results are supported by direct measurements of IR from glycogen-depletion studies that showed that IR is directly correlated with unit tetanic force in normal (Fig. 3.12; Bodine et al. 1987; Chamberlain and Lewis 1989; Tötösy de Zepetnek et al. 1992) and reinnervated muscles (Fig. 3.12; Tötösy de Zepetnek et al. 1992).

Several studies in self- (Bagust and Lewis 1974; Gordon and Stein 1982a) and cross-reinnervated muscles (Bagust et al. 1981; Gordon et al. 1986;1988) have shown that electrophysiological measures of axon size are well correlated with unit force. In addition we have shown that the increase in unit force in partially denervated muscles is also size dependent (Chapter 2). The present results, therefore, extend these findings to include size dependent branching in muscles reinnervated after nerve crush or nerve transection with N-N repair where MUs were forced to increase in size to compensate for the reduced MU number.

What factors prevent the reestablishment of the normal size relationship in muscles reinnervated after N-M suture when the number of MUs was severely reduced? The loss of the size relationship can be attributed to two characteristic changes that occurred in muscles reinnervated after N-M suture when the number of MUs was reduced by more than 50%. First, the size of the FG fibers decreased to become similar in size to SO and FOG fibers (Fig. 5.7) and second, the IR of FR and FF units did not increase by the same extent as the normally smaller S units (discussed in detail in Chapter 3). As a result, the normal differences in mean unit force of S and FF units was dramatically reduced and the overall range in unit force decreased (Fig. 5.10). Therefore, even if there is some relationship between axon size and IR, the correlation is obscured by the small range in unit force and axon potential amplitude.

Size dependent branching of motoneurons appears to be a general trait of developing and regenerating motoneurons. Even when the largest motor axons cannot expand their unit territory to innervate a larger number of muscle fibers than normal, as is the case after N-M suture, motoneurons still branch in a size dependent fashion as evident by the consistently larger F units compared to S units in all reinnervated muscles.

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6. DETERMINATION OF THE RANGE IN MOTOR UNIT INNERVATION RATIO IN THE CAT MEDIAL GASTROCNEMIUS MUSCLE BY LONG-TERM ELECTRICAL STIMULATION

6.1 INTRODUCTION

Slowing of fast-twitch skeletal muscle, as a result of chronic stimulation, is associated with a decline in force (Eerbeek et al. 1984; Pette et al. 1976; Salmons and Henriksson 1981). Decline in force, in turn, is associated with reduced muscle fiber size (Donselaar et al. 1987). While fast to slow phenotype transitions have been the subject of many studies (reviewed by Pette and Vrbova 1985), the effects of chronic stimulation on muscle force has been relatively neglected (Kernell et al. 1987a).

There is normally a wide range in motor unit (MU) force in most skeletal muscles. Differences in unit force have been attributed to differences in size of muscle unit fibers and the intrinsic force per unit area (specific force; SF) (Burke 1981; Stein et al. 1990). In addition to muscle fiber cross-sectional area (CSA) and force per unit CSA, the product of which is force per fiber, the number of fibers per motoneuron (innervation ratio: IR) must also be considered.

Recent glycogen-depletion techniques (Edstrom and Kugelberg 1968) have been used to show that in cat tibialis anterior (TA) (Bodine et al. 1987), rat TA (Tötösy de Zepetnek et al. 1992), and soleus muscles (Chamberlain and Lewis 1989), differences in IR make a significant contribution to the 10- fold range in unit force. Larger pinnation angles of many muscles, such as that cat medial gastrocnemius (MG), increases their architectural complexity such that enumeration of glycogen-depleted fibers becomes more difficult (Burke and Tsairis 1973; Chamberlain and Lewis 1989). For this reason, Burke and Tsairis (1973) limited their sample of glycogen-depleted MUs in the cat MG to 4 and instead devised an indirect method for determining the relative contribution of IR, CSA and SF to the range in unit force. They physiologically classified MUs as slow (S), fast-fatigue resistant (FR) and fast-fatigable (FF) and muscle fibers as Type I, Type IIA, and Type IIB based on histochemical criteria (Burke et al. 1973). Since there is reasonable correspondence in glycogen-depleted MUs (Burke et al. 1973) they used the number of MUs and corresponding number of muscle fibers or each type to calculate the relative IR of each MU type (the ratio of muscle fibers to MU numbers). By comparing the relative IR, mean CSA, and calculating the mean SF for each MU type these authors found that differences in CSA and SF accounts for the differences in mean unit force where S<FR<FF. IR was not considered to differ sufficiently to account for the force difference between different MU types. However, the indirect method of determining IR, the relative contributions of CSA, and SF does not take into account differences in mean fiber size between individual MUs of the same type (Bodine et al. 1987; Tötösy de Zepetnek et al. 1990) nor the overlap in CSA of fibers of different types. Both these may underestimate the contribution of IR with a corresponding overestimate of the contribution of SF (eg. Gordon et al. 1988). Furthermore, inadequate sampling of either MUs or muscle fiber types can severely distort the calculated relative IR (see Discussion in Stein et al. 1990; Tötösy de Zepetnek

et al. 1992).

As a result, the relative contribution of IR, CSA and SF in determining unit force in large complex muscles such as the MG remains unclear. The evidence for a major role of IR in determining unit force, obtained from simpler and smaller TA (Bodine et al. 1987; Tötösy de Zepetnek et al 1992) and soleus muscles (Chamberlain and Lewis 1989), does not necessarily apply to more complex muscles. Interestingly, the range of fiber size in the MG muscle is not substantially higher than in the TA. Each muscle also has the same fiber types, yet the range the in unit force is 10 times greater in the MG than TA. Intuitively, this comparison suggests that either 1) the difference in mean muscle fiber CSA between different MUs is higher in large MG muscles, 2) SF differs more and/or, 3) there is a wider range in IR to account for the greater range in force in these large muscles.

Since low-frequency, high duration electrical stimulation converts muscle fibers to slow-oxidative (SO) (reviewed by Pette and Vrbova 1985) and reduces fiber size to a narrow range (Brown et al. 1976; Donselaar et al. 1987; Pette et al. 1975; Salmons and Henriksson 1981), the chronically stimulated muscle provides a very nice model to examine the relative contribution of IR to the wide range in force in large complex muscles. Since stimulation reduces the range of CSA, and muscle fibers are converted to the slow phenotype, any remaining range in unit force in a chronically stimulated muscle can be attributed to a range in IR which is unlikely to be affected by the stimulation. Unit tetanic force is a reliable index of IR provided that the other factors contributing to force, namely the average muscle fiber CSA and SF are not significantly different between MUs. The present studies were undertaken to test this hypothesis and results are compared with direct estimations of IR from glycogen-depleted MUs in normal cat MG muscles (results from Chapter 3).

A preliminary account of the results have been published in abstract form (Rafuse et al. 1991a;1992; Pattullo et al. 1992).

6.2 METHODS

Six adult cats were used in this study. Ampicillin (10 mg/kg) was given subcutaneous one hour prior to surgery. Cats were anesthetized with an intraperitoneal injection of 40 mg/kg sodium pentobarbital (Somnotol). Under strict aseptic conditions an incision was made along the dorsum of the right hind limb to expose both the MG muscle and nerve to place stimulating electrodes either intramuscularly near the MG nerve or around the nerve in a nerve cuff. In the first 2 cats, insulated Cooner wires (AS632) were inserted into the muscle near the point of nerve entry. Wire insulation was removed from a small area (5 mm) to expose the bare wire just under the surface of the muscle. At least 3 electrodes were inserted into the muscles around the nerve at a distance of 3-5 mm. The pair of electrodes giving the lowest threshold for muscle activation was used for chronic stimulation. However, over time the current required to evoke maximum force and EMG had to be increased, presumably because intramuscular electrodes migrated from the original insertion points (Popovic et al. 1991). Therefore, for the next 4 cats, a nerve cuff electrode was developed to stimulate the MG muscle selectively.

The MG nerve cuff electrodes were constructed from 3 Cooner wires (AS632) sewn through reinforced silastic (Dow Corning, 0.007 mm) sheets (10 mm^2). Three 6-0 silk sutures were fastened to the silastic sheet with Medical Grade silicone in order to secure the sheet in a cuff-like position when wrapped around the nerve. The nerve cuff construction was well suited to the cat MG nerve since it was both small and flexible thereby reducing any risk of nerve damage during normal limb movement.

The nerve cuff was gently placed around the MG nerve after freeing the nerve from connective tissue just proximal to its entry point into the muscle. The nerve cuff was placed close to the muscle to ensure that natural movement of the limb would not damage the nerve. A patch bipolar EMG electrode was fastened to the fascia on the medial midbelly of the muscle with 4-0 silk sutures. All wires were fed subcutaneously from the hind limb to exit through an incision made in the cat's back along the lower lumbar region. The percutaneous electrode wires were fastened to an IC connector which, in turn, was connected to the small portable stimulator. The stimulator was mounted on a saddle constructed out of plaster of paris (Hexelit) which was secured to the cats back between S1-L7 and L6-L5 vertebrae with #1 prolene sutures. In 1 control cat, the percutaneous wires was simply left extended so that they could be later connected to a stimulator and amplifier for weekly measurements of muscle contraction (see below). Following surgery cats were administered additional injections of ampicillin and bupromorphine (0.01 mg/kg) to reduce risk of infection and discomfort. Cats were housed in large cages which permitted normal walking and playful activities. The operated cats showed no signs of discomfort or even awareness of the saddle and stimulator fastened to their backs.

MG muscles were chronically stimulated with a small (80 g) battery powered stimulator that could easily be carried on the saddle. The stimulator was designed and constructed locally by D. Charles and Z. Kenwell. The stimulator produced trains of 20 pulses per second for 2.5 seconds followed by 2.5 seconds with no stimulation (50%

duty cycle). This pattern of stimulation was carried out 24 hours per day for between 42 and 240 days. The stimulator produced a balanced bi-phasic stimulating pulse to prevent any potentially damaging charge build-up around the electrodes next to the nerve. Pulse-width could be adjusted to vary the strength of stimulation. Stimulating nerve cuff electrodes provide a wide margin of stimulus strength prior to spread of current from the cuff to surrounding nerves (Popovic et al., 1991) and therefore maximal stimulation of the MG muscle was easily identifiable and ensured.

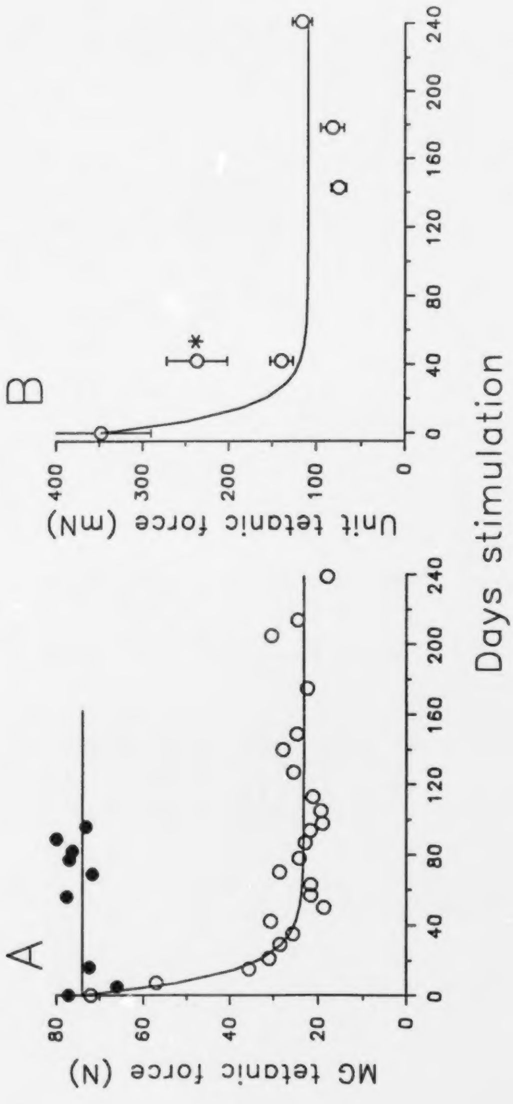
At regular intervals, cats were anesthetized with halothane and the foot placed in a specially designed boot that was coupled to a force transducer (Grass FT10) for isometric force recordings evoked by stimulation of the MG nerve. The length of the muscle was adjusted by moving the boot along the line coaxial with the ankle joint in order to obtain the optimal length for maximum isometric force. The MG nerve was stimulated by coupling the electrode wires to a stimulator (Bell) to evoke EMG, twitch and tetanic contractions. EMG and force responses were averaged using a PDP 11/21 microcomputer and stored on disk. Since each animal served as its own control, force measurements are shown in Newtons (N; Fig. 6.1A) and not corrected for the lever arm and expressed as torque measurements.

The MG muscle was stimulated for between a 42 to 240 day period after which a final acute experiment was performed to physiologically characterize a large sample of MUs (31-56) and to repetitively stimulate a single MU to deplete its muscle fibers of glycogen. MG muscles in both hindlimbs were removed for histochemical analysis and for measurements of depleted and non-depleted muscle fiber CSAs.

The experimental procedures used for preparation and physiologically recording isolated MUs, glycogen-depletion of a single MU, and histochemical analysis of muscle fibers, and statistical analysis have been described in detail in Chapter 3.

Figure 6.1.

Chronic measurements of isometric force recorded from 1 stimulated (open circles) and 1 non-stimulated MG muscles (filled circles; A). The force of the stimulated muscle decreased rapidly to approximately 1/3 its original value recorded immediately after implantation of MG nerve cuff electrodes. Force of non-stimulated muscles did not change after implantation of nerve cuff electrodes. Decline in force of stimulated muscle is well fitted to a double exponential decay curve. B. Mean (± S.E.) unit tetanic force recorded acutely following 42 to 240 days stimulation. Mean and range in unit force decreased with a similar time course as the fall in whole muscle force. Mean unit force was significantly higher in 1 muscle (indicated by asterisks) stimulated for 6 weeks followed by 2 weeks of no stimulation.


A
MG tetanic force (N)
80
60
40
20
0
0
40
80
120
160
200
240
B
Unit tetanic force (mN)
400
300
200
100
0
0
40
80
120
160
200
240
*
Days stimulation

6.3 RESULTS

Synchronous activation of all muscle fibers in cat MG muscles by chronic electrical stimulation at 20Hz in a 50% duty cycles (2.5 sec. on, 2.5 sec. off) leads to a dramatic fall in muscle force as illustrated in Fig. 6.1A. The same nerve cuff or intramuscular electrodes used to stimulate the MG muscle in conscience cats was used under Halothane anesthesia to evoke EMG and contractile responses. Twitch and tetanic contractions were recorded at lengths where isometric force was maximal. Immediately after implantation the tetanic force averaged (± S.E.) 62.4 ± 9.1 N. Force remained constant if the muscle was not stimulated as indicated by the stable force recorded from 1 MG muscle implanted with a nerve cuff, but not used to chronically stimulate the muscle (Fig. 6.1A; filled circles). The force of all stimulated muscles decreased to approximately one third its original value as shown for 1 stimulated muscle in Fig. 6.1A (open circles). Regular recordings showed that force fell within the first month of stimulation following a double exponential decay with time constants of 12.7 and 3.0 X 10^{11} days.

Twitch and tetanic force was recorded in a large sample of MUs during the plateau phase, namely at 42, 143, 180, 240 days after initiating chronic stimulation (Fig. 6.1B). The arithmetic mean of unit tetanic force was a third of the mean value of normal unstimulated muscles. The fall in mean unit force follows a similar time course to the decline in whole muscle force. The decline may be reversed within two weeks if stimulation is discontinued as indicated by the larger mean unit force in 1 muscle that was stimulated for 6 weeks followed by 2 weeks of non-stimulation (indicated by asterisk in Fig. 6.1B). Note that the standard error bars of the arithmetic means decreased dramatically after stimulation indicating a reduced variance around the mean.

Unit tetanic forces and fatigue indices normally vary over a wide range in cat MG muscles (Fig. 6.2A; see also Burke et al. 1973). In Fig. 6.2, S units are distinguished from F units by using different symbols and F units are subdivided into FR, FI, and FF by their fatigue indices of >0.75, 0.25 - 0.50, and <0.25, respectively. Normally, the weakest MUs are S units which are resistant to fatigue (fatigue index >0.75) while the most forceful MUs are fatigable with fatigue indices <0.25. After 42 days stimulation, all 49 MUs sampled were resistant to fatigue and the range in unit force was decreased primarily because the most forceful MUs were lost (Fig. 6.2B; Table 6.1). There were no FI or FF units in this stimulated muscle.

The distribution of unit force, in the cat MG muscle, is skewed to the right due to the larger proportion of small as compared to large forceful units (Burke 1981). On a logarithmic force axis, which represents the smaller units more equally, unit force is bimodally distributed and varies over a 100- fold range (Fig. 6.3A). The smallest MUs are predominantly S units (open histograms), the intermediate force producing MUs are FR units (cross-hatch histograms) and the most forceful units are FF units (filled histograms) as shown in Fig. 6.3A and Table 6.1. After 42 days stimulation, the geometric mean and range in unit tetanic force decreased by approximately 50% to become 96 mN and 35- fold, respectively. The bimodal distribution was maintained, but the second mode narrowed as the largest MUs became less forceful (Fig. 6.3B),

Figure 6.2.

Fatigue index of MUs in normal (A) and muscle stimulated for 42 days (B) plotted as a function of their unit tetanic force (mN). Fast (open squares) and slow (filled circles) are shown. Horizontal lines divide fast units into FR (fatigue index >0.75), FI (index >0.25 and <0.75) and FF (index <0.25) units. No FI or FF units were physiologically characterized in stimulated muscles.

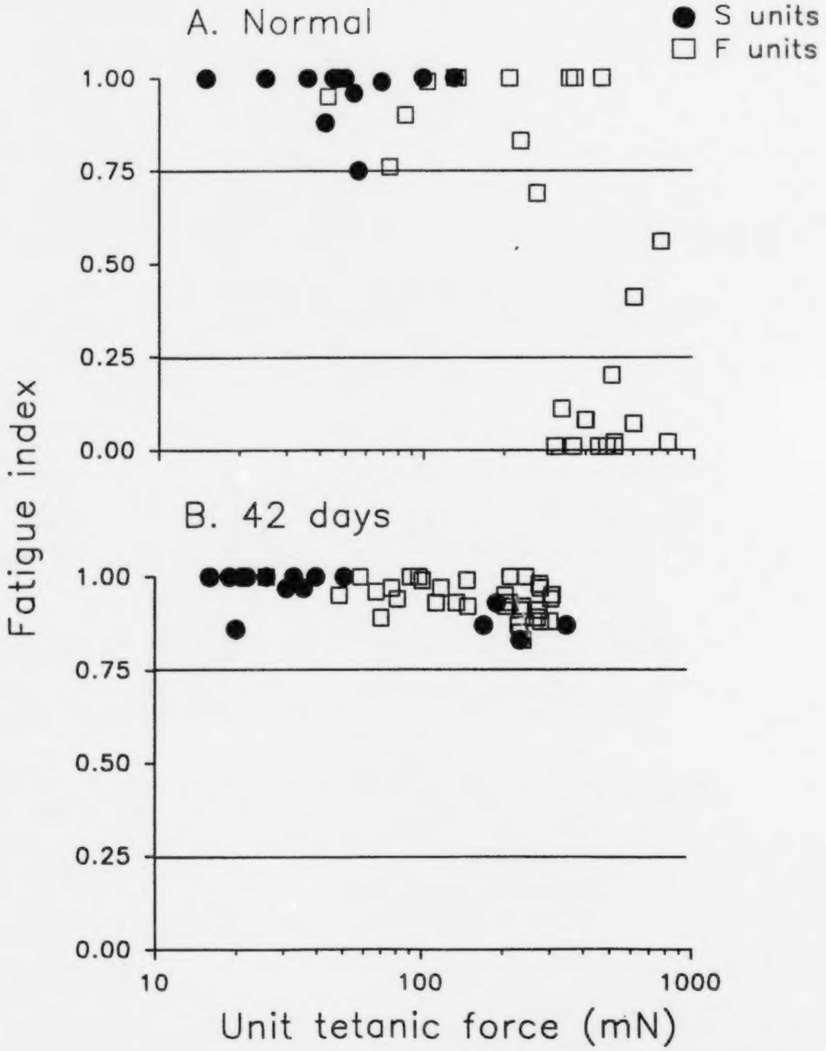

A. Normal
● S units
☐ F units
1.00
0.75
0.50
0.25
0.00
Fatigue index
B. 42 days
1.00
0.75
0.50
0.25
0.00
10
100
1000
Unit tetanic force (mN)

Figure 6.3.

Frequency histogram of unit tetanic force from normal (A) and muscle stimulated for 42 days (B). MUs of different types are identified with different histogram shading. Following 42 days stimulation the normal 100- fold range in force decreased to 35- fold due to selective loss of the largest force producing MUs. No FI or FF units were physiologically characterized in the stimulated muscles.

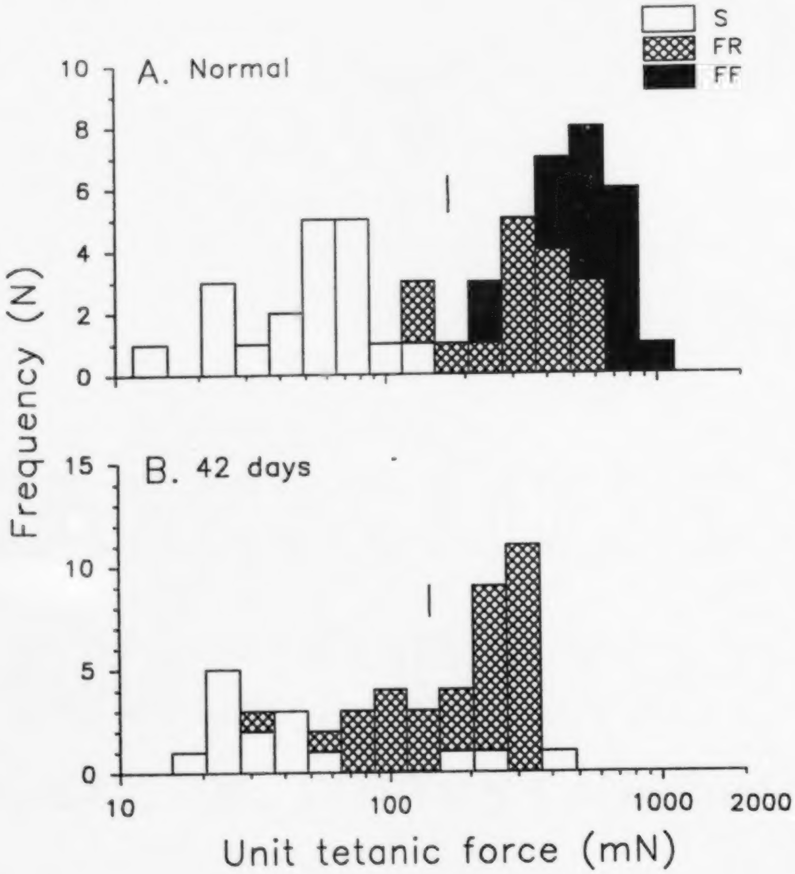

S
FR
FF
A. Normal
10
8
6
4
2
0
Frequency (N)
B. 42 days
15
10
5
0
10
100
1000
2000
Unit tetanic force (mN)

Table 6.1. Summary of physiological MU types and histochemical muscle fiber types in normal and stimulated MG muscles.

	SO CSA	FOG CSA	FG CSA	S unit force	FR unit force	FI unit force	FF unit force
Normal	2839 ± 58	2750 ± 71	6156 ± 107	79 ± 14	239 ± 25	520 ± 66	598 ± 38
42 d	2491 ± 33	1967 ± 31	2530 ± 39	73 ± 25	148 ± 15	-	-
143 d	1641 ± 30	-	-	75 ± 39	-	-	-
240 d	3127 ± 23	-	-	116 ± 12	-	-	-
	% SO	% FOG	% FG	% S	% FR	% FI	% FF
Normal	26 %	22 %	46 %	25 %	25 %	3 %	47 %
42 d	40 %	34 %	26 %	31 %	69 %	-	-
143 d	97 %	3 %	0 %	100 %	-	-	-
240 d	98 %	2 %	0 %	100 %	-	-	-

Values are mean ± S.E. Fiber CSA in μm^2 and unit tetanic force in (mN).
SO, slow oxidative; FOG, fast-oxidAtive glycolytic; FG, fast glycolytic; S, slow unit; FR, fast-fatigue resistant; FI, fast-fatigue intermediate; FF, fast fatigable.

concurrent with the increase number of S and FR units (Fig. 6.2B and Table 6.1).

The apparent loss of MUs with tetanic forces >500 mN was absorbed by an increase in number of FR units developing forces in the range of 100 to 300 mN (Fig. 6.3B). This change was accompanied by a parallel loss of muscle fibers with CSA greater than 5 X 10^3 μm^3 (cf Fig. 6.4A and B). At this time (42 days of stimulation), the mean size and range in size of FG, FOG, and SO fibers were the same and equal to the normal size of SO fibers (see below). Even though 26% of the muscle fibers were still classifiable as FG fibers according to their mATPase activity, no FF units were found in this stimulated muscle.

Thus, stimulation leads to a rapid change in muscle fiber size (within the first month) which precedes the phenotypic change from fast to slow (ie. FG and FOG to SO fibers). At 1 month, the number of SO fibers increased relative to FG fibers (see Table 6.1) showing partial conversion of muscle fiber types at this time. Therefore, change in fiber size is one of the earliest changes in muscle fiber properties. Because stimulation is unlikely to affect innervation, and therefore the number of muscle fibers per motoneuron (IR), the reduced range in unit force, from 100- to 35- fold, is attributed to the reduced muscle fiber size as previously suggested (Donselaar et al. 1987).

Mean unit force and muscle fiber size did not change significantly between 40 and 240 days of stimulation as shown by the population distributions of unit force (Fig. 6.5A, inverted histogram) and muscle fiber CSA (Fig. 6.5B, inverted histogram) from all three muscles stimulated from 143 to 240 days. During this time (40 to 143 days) fast to slow fiber conversion progressed slowly such that all fibers are histochemically

Figure 6.4.

Frequency histograms of muscle fiber CSA measured from normal (A) and muscle stimulated for 42 days (B). Muscle fibers of different types are identified with different histogram shading. Distribution of fiber CSA in normal muscles is bimodal and becomes unimodal following 42 days stimulation such that the second mode is lost. The mean and range of all 3 fiber types in stimulated muscles are similar to those of normal SO fibers.

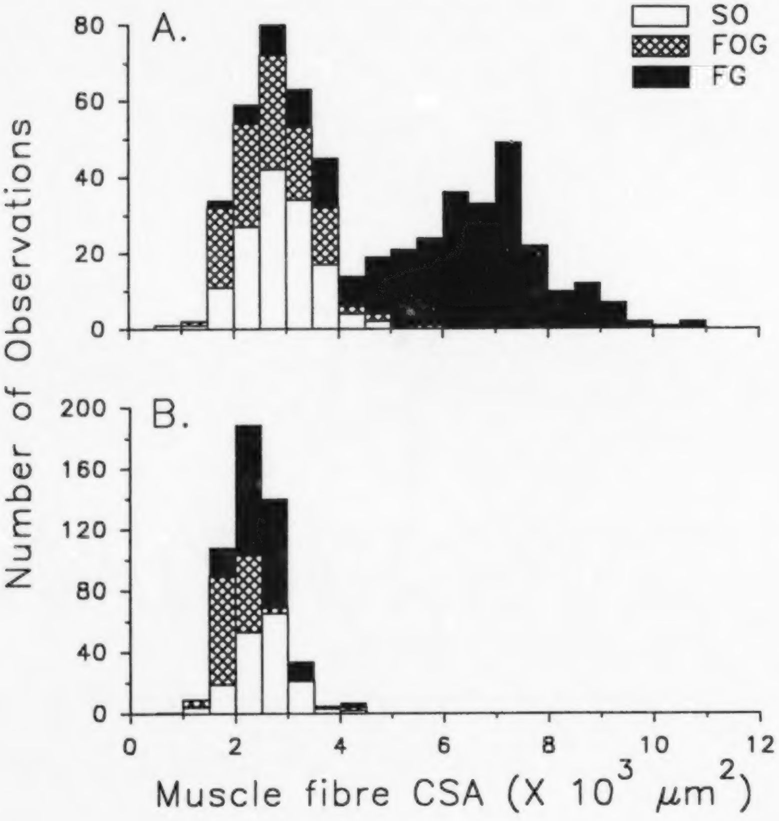
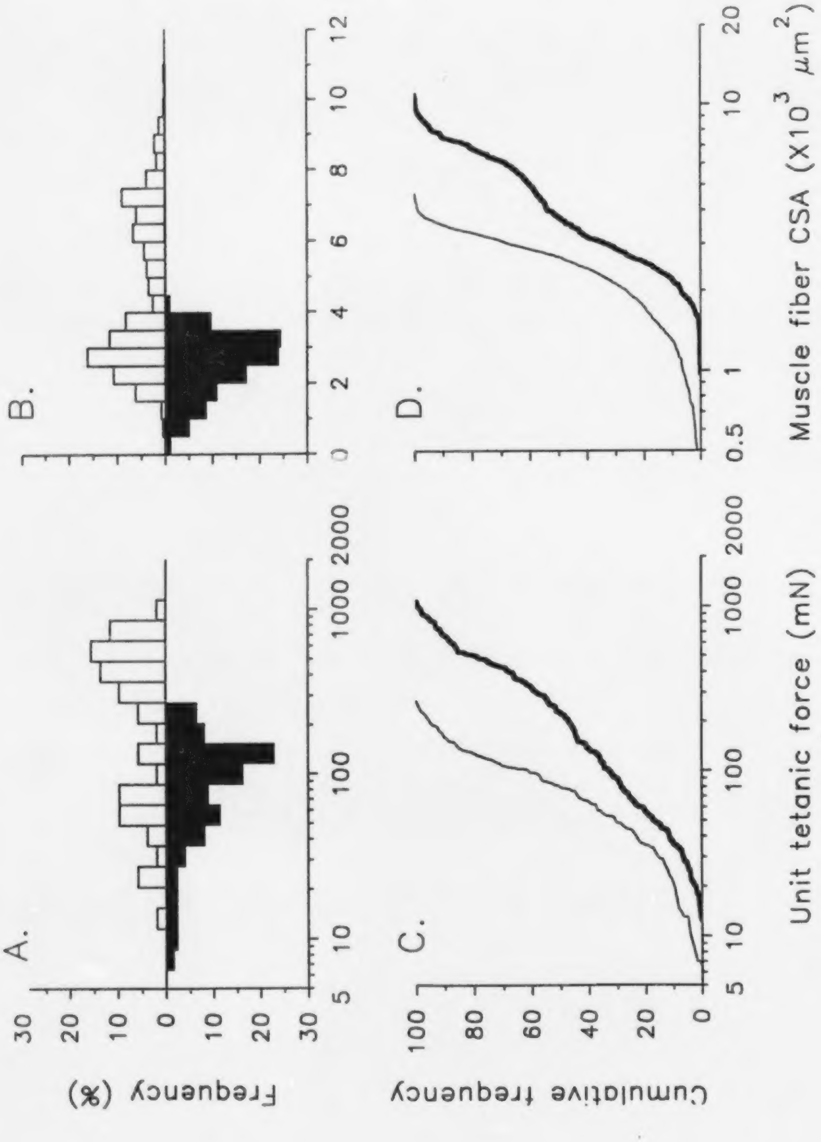

A.
B.
SO
FOG
FG
Number of Observations
Muscle fibre CSA (X 10^3 μm^2)
0
20
40
60
80
0
40
80
120
160
200
0
2
4
6
8
10
12

Figure 6.5.

Comparison of frequency histograms of unit tetanic force (A) and muscle fiber CSA (B) from 2 normal muscles (open histograms) and 3 muscles stimulated for ≥143 days (filled histograms). C,D are cumulative frequency histograms of unit tetanic force and muscle fiber CSA for values plotted in A and B, respectively, and plotted on logarithmic X-axes. Normal distributions are denoted by thicker lines in C,D. Non-parallel shift in both cumulative distributions indicate that the stronger force producing MUs and larger muscle fibers decreased more than weaker MUs and smaller fibers.


A.
B.
C.
D.
Frequency (%)
30
20
10
0
10
20
30
5
10
100
1000
2000
0
2
4
6
8
10
12
Cumulative frequency
100
80
60
40
20
0
5
10
100
1000
2000
0.5
1
10
20
Unit tetanic force (mN)
Muscle fiber CSA (X10³ μm²)

classifiable as SO fibers by 143 days stimulation (Table 6.1).

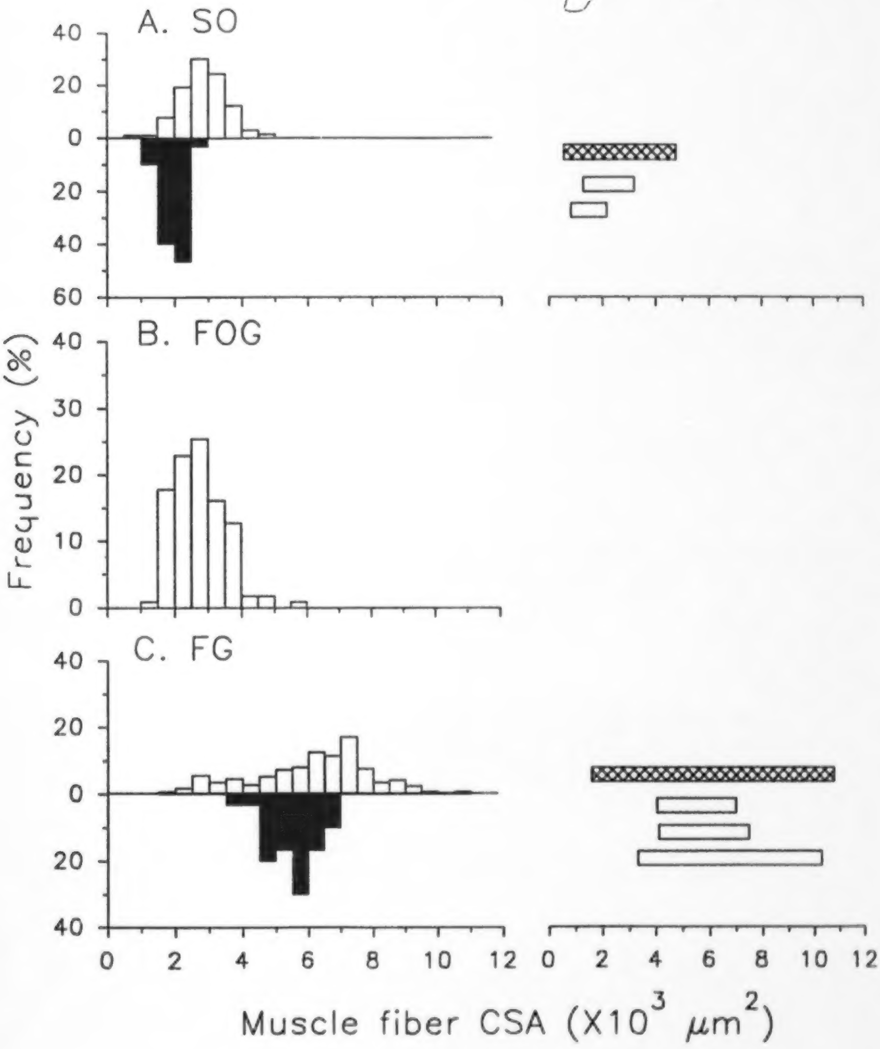
Although there was a small difference in animals, presumably as a result of different animal size (cats weighed between 3.0 and 4.9 kg; mean ± S.E.: 3.5 ± 0.4 kg), the mean unit force and fiber size was very similar for the 3 MG muscles stimulated for between 143 and 240 days. The data from all 3 animals is pooled (inverted histograms, Fig. 6.5) for comparison with the data from 2 normal cats (open histograms, Fig. 6.5). Clearly the range of unit force and fiber size are smaller, but can the change in force simply be explained by the reduction in fiber size? To answer this question, Figs. 6.5A and 6.5B are replotted as cumulative frequency histograms on logarithmic X-axes (Fig. 6.5C and D, respectively). Normal distributions are indicated by the thicker lines in the figure. Comparison of the shift to the left in the force and fiber size shows that at the lower end of the curve, the shift is comparable, but that the decrease in force for the upper 40% of the MU population is larger than the corresponding shift of the same population of muscle fibers. The additional factor that is likely to contribute to the decline in force is SF which is presumably reduced as F units are converted to S units. After 140 days of stimulation all MUs were classified as S units on the basis of their twitch contraction times (>40 ms), absence of sag and fatigue indices (>0.25) and all fibers were classified as SO based on their mATPase reactivity (Table 6.1).

6.3.1 Muscle unit fiber size

Muscle fibers within a single MU normally vary in size, but clearly do not cover the range of the non-unit fibers of the same type (Fig. 6.6). The distribution of muscle

Figure 6.6.

Frequency histogram distributions of nondepleted muscle fiber CSA of SO, FOG, and FG (A-C, open histograms). In A and C the distribution of nondepleted fiber CSA are compared with the distribution of muscle fiber CSAs within glycogen-depleted MUs containing muscle fibers of the same histochemical type (filled histograms). Box plots compare the range in fiber CSA of non-depleted SO and FG fibers (Cross-hatched boxes) with the range of SO and FG fiber size within 2 single S-units and 3 single FF-units (open boxes, A and C, respectively).

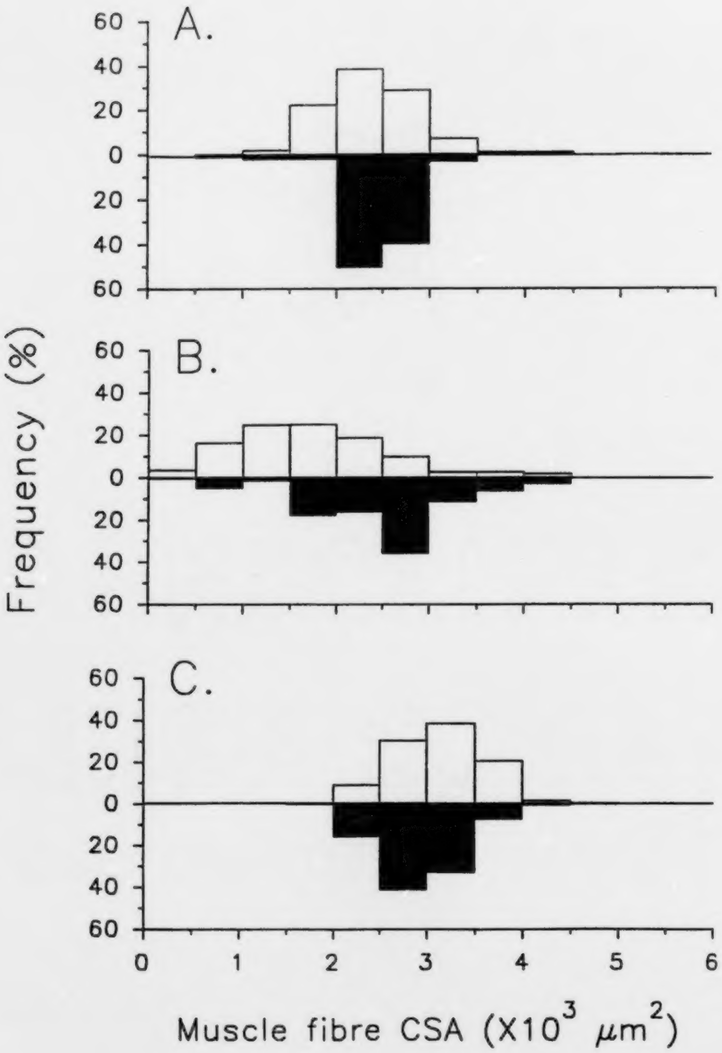

A. SO
B. FOG
C. FG
Frequency (%)
40
20
0
20
40
60
40
30
20
10
0
40
20
0
20
40
0
2
4
6
8
10
12
0
2
4
6
8
10
12
Muscle fiber CSA (X10^3 μm^2)

unit fiber size is plotted as the mirror image (lower image) of the non-depleted fibers for convenience. The box plots besides the frequency distributions in Fig. 6.6 show the range in fiber CSAs from the 2 glycogen-depleted S and 3 FF units (open boxes) are compared with the range in non-depleted muscle fibers of the same type, measured in the same muscle (cross-hatched boxes). Although the smaller range in muscle unit fiber size contrasts with the wide range in fiber size within the whole population of the same type, the small difference in CSA of muscle fibers between MUs cannot account for the range in force observed within that MU type. For example, a 2-fold difference in the mean CSA between S units cannot account for the 10- fold range in S unit force. Similarly, differences of 3 to 4- fold in the mean muscle fiber CSA of FF muscle units cannot account for the normal 10- fold range in FF unit force (Burke 1981; Gordon and Stein 1982).

After 42 day of stimulation the size of fibers within 1 isolated MU spanned the same range as all non-unit fibers (Fig. 6.7A). Consistent results were obtained for glycogen-depleted single MUs in muscles after 143, and 240 (Fig. 6.7B and C, respectively). Therefore, the 35- fold range in unit force must be attributed to differences in SF and/or IR. After 143 days stimulation, when all fibers are SO and the SF values between muscle fibers were presumably the same, the 35- fold range in unit force can be attributed directly to differences in IR of the different MUs.

Figure 6.7.

Frequency histogram distributions of fiber CSA from non-depleted muscle fibers (open histograms) and glycogen-depleted (filled histograms) single muscle unit fibers in muscles stimulated for 42 (A), 143 (B) and 240 days (C). Mean and range of depleted fibers are similar to that of non-depleted fibers in all 3 muscles.


A.
B.
C.
60
40
20
0
20
40
60
Frequency (%)
0
1
2
3
4
5
6
Muscle fibre CSA ($\times 10^3$ μm^2)

6.3.2 Determination of IR from direct measurements of glycogen-depleted muscle fibers.

Counting all glycogen-depleted muscle fibers within a single MU in the cat MG muscle is difficult due to the steep pinnation of muscle fibers. It is therefore impossible to visualize all unit fibers in a single muscle cross-section. Nevertheless, if a number of serial cross-sections are cut at several proximodistal levels along the length of the muscle, one section will contain the highest number of depleted fibers corresponding to the center of the unit territory (see Chapter 4). If it is assumed that all unit territories have the same general shape, counting the depleted muscle fibers through the center of the territory will provide a reasonable estimate of the unit IR (Burke and Tsairis, 1973).

The counts of isolated and characterized MUs in normal and stimulated muscles are shown in Table 6.2 and the normal values plotted as a function of unit tetanic force in Fig. 6.8. Although the tetanic force of the 5 normal MUs sampled only varied between 151 to 416 mN, the Y-axis is drawn to represent the normal range in MG unit tetanic force. The X-axis is increased correspondingly. Force increases as a function of IR; the slope (± S.E.) of the regression line being 0.91 ± 0.23 (RO: 0.92). If we extrapolate the line from the smallest to largest normal MUs sampled, namely 15 mN and 1083 mN, respectively, it can be seen that IR ranges between 20 and 1000 fibers which is a 50- fold range. This extrapolated range in IR is in reasonable agreement with the 35- fold range in unit force remaining in chronically stimulated muscles where SF and CSA of muscle unit fibers were eliminated as determinants of unit force.

Figure 6.7.

Frequency histogram distributions of fiber CSA from non-depleted muscle fibers (open histograms) and glycogen-depleted (filled histograms) single muscle unit fibers in muscles stimulated for 42 (A), 143 (B) and 240 days (C). Mean and range of depleted fibers are similar to that of non-depleted fibers in all 3 muscles.

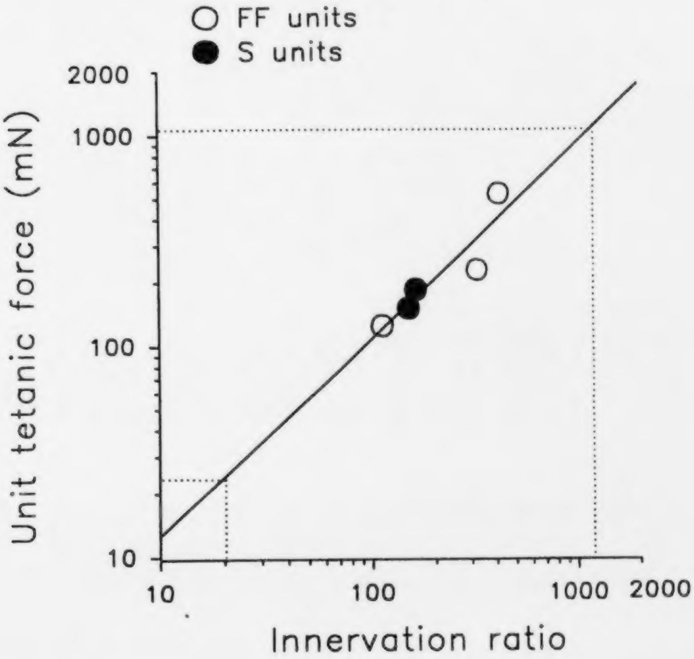

○ FF units
● S units
Unit tetanic force (mN)
2000
1000
100
10
10
100
1000
2000
Innervation ratio

Table 2. Summary of the glycogen-depleted MU data

<table>
<tr><th colspan="4">NORMAL</th></tr>
<tr><th>Type</th><th>Force, mN</th><th>Innervation Ratio</th><th>Muscle Fiber CSA μm^2</th></tr>
<tr><td>SO</td><td>150</td><td>151</td><td>1261 ± 30</td></tr>
<tr><td>SO</td><td>185</td><td>163</td><td>2025 ± 40</td></tr>
<tr><td>FG</td><td>528</td><td>416</td><td>6538 ± 394</td></tr>
<tr><td>FG</td><td>125</td><td>134</td><td>5556 ± 131</td></tr>
<tr><td>FG</td><td>229</td><td>325</td><td>5709 ± 92</td></tr>
<tr><th colspan="4">STIMULATED</th></tr>
<tr><td>SO</td><td>75</td><td>151</td><td>2533 ± 96</td></tr>
<tr><td>SO</td><td>117</td><td>117</td><td>2927 ± 39</td></tr>
<tr><td>SO</td><td>139</td><td>309</td><td>2435 ± 39</td></tr>
</table>

Mean values ± S.E.
SO, slow oxidative; FG, fast-glycolytic

6.4 DISCUSSION

A combination of chronic recording techniques with analysis of single MUs at selected times during chronic low frequency, long-term stimulation, have in this study provided 1) important insights in the time course of change in muscle fiber size and phenotype and 2) very strong evidence that IR is the most important factor regulating the force of MUs in muscles. By increasing the range in IR in a muscle, the range of unit force is increased dramatically without necessitating a very broad range of muscle fiber size in different skeletal muscles.

6.4.1 Time course of change in muscle fiber size and phenotype.

In agreement with other studies of low frequency, high duration stimulation of rabbit and cat muscles (Salmons and Vrbova 1969; Donselaar et al. 1987; Pette et al. 1986; Salmons and Henriksson 1981), stimulated cat MG muscles developed less force than normal and muscle fiber size decreased. Using chronic recording techniques developed in our labs, our results from stimulated MG muscles show that whole muscle tetanic force decreased rapidly, following a double exponential curve, to reach a stable force value equal to approximately 30% its original value (Fig. 6.1A). The reduction in mean unit tetanic force was reasonably fitted by the same exponential curve and decreased by the same factor of 70% (cf. Fig. 6.1A,B). There was a concurrent decrease in the size and range in muscle fiber CSA and increase in resistance to fatigue consistent with previous findings (Donselaar et al. 1987; Kernell et al. 1987). The more rapid change in muscle force and endurance (and associated muscle fiber size) compared to the change in muscle fiber phenotype is readily seen in this study. It was very striking that the change in force and fiber size reached a plateau by 42 days after onset of stimulation where mATPase staining still revealed all 3 muscle fiber types (ie. FG,FOG,SO). The absence of FF and FI units at 42 days of stimulation, when FG fibers are classified on the basis of mATPase, is consistent with physiological studies that showed muscle endurance increases more rapidly than slowing of muscle contraction (reviewed by Pette and Vrbova 1992). These results are also consistent with histochemical analyses that show increase in oxidative relative to glycolytic potential occurs prior to an increase in slow myosin heavy and light chain expression (see Pette and Vrbova 1992 for references). Upregulation of slow forms of mRNA level is more rapid, but the slow rate of turnover of the contractile proteins contributes to the slower conversion of muscle fiber phenotype (Pette and Vrbova 1992).

The factors regulating the switch in gene expression in response to low frequency high duration activity are not yet understood. The more rapid change in fiber size than change in phenotype indicates that increased activity rapidly changes the protein content of the muscle cells. However, the relative contribution of reduced synthesis and increased breakdown to the reduced fiber size in response to dramatic stimulation is still unclear. The relative contribution is different in different models of atrophy (reviewed by Gordon and Pattullo 1992). Many authors have identified altered energy supply as the trigger for changes in fiber size and phenotype (reviewed by Pette and Vrbova 1992).

Normally, the most frequently recruited and active muscle fibers are the smallest.

Unit force, fiber size, and endurance are inversely correlated with recruitment order; namely FF > FI > FR > S and FG > FOG > SO (Henneman and Mendell 1981; Henneman and Olson 1965). The small CSA of the most active fibers is presumably important in facilitating the diffusion of oxygen from the capillaries to the center of the muscle fibers (Salmons and Henriksson 1981; Pette and Vrbova 1985). Clearly, activation of muscle fibers by electrical stimulation for the equivalent of 12 hours per day, as used in this study, imposes a large metabolic demand on the contracting muscle fibers. The results of this study indicate that the earliest compensatory changes observed in the largest FG fibers from FF units is to decrease their diameter to that of normal SO fibers and increase their oxidative capacity in order to adapt to the increased energy demand.

While there is normally good correspondence between the physiological and histochemical profiles of muscle units (Burke et al. 1973) our findings that there is a period of mismatch between MU and histochemical fiber types during the initial period of muscle fiber type transformation is consistent with the different time course of change in the physiological parameters of force, endurance, and contractile speed and their corresponding structural and enzymatic correlates, fiber size, metabolic enzymes, and contractile proteins. For example, conversion of phenotype involves several transitions from type FOG to SO during which time hybrid fibers are common with slow and fast isoforms of myosin heavy chains (MHC) coexisting within the same fibers after 6 (Pette and Schnez 1977) and 20 weeks stimulation (Pattullo et al. 1992), but only slow MHC after 240 days stimulation in cats (Pattullo et al. 1992).

Force recovers rapidly after discontinuing chronic stimulation as shown by the recovery of unit force towards normal within 2 weeks. (indicated by asterisks in Fig. 6.1B). This finding supports the results of Kernell and Eerbeek (1991) who showed that, in cat peroneus longus muscle, force recovers rapidly within 4 weeks after a 4 week period of 40 Hz stimulation using a 50% duty cycle. The normal fatigue resistance and twitch contraction time returned more slowly (Kernell and Eerbeek 1991) consistent with the rate of change in force, endurance and speed during stimulation. Rate of recovery of muscle properties therefore provides a reasonable means of studying the rate limiting steps during fiber transitions (Pette and Vrbova 1992).

6.4.2 Determinants of force in large complex muscles

Unit force is the product of 3 factors: mean muscle unit fiber CSA, SF, and IR (Burke 1981). The range within each of the 3 factors determines the overall range in unit force (Stein et al. 1990). For example, if each factor varies over a 2-fold range then the overall range in unit force is 8- fold (ie. 2 * 2 * 2). Thus, the overall range in unit force is determined by the relative contribution of each of the 3 factors. In this study, where we have studied chronically stimulated muscles at the single MU level for the first time, our results demonstrate a wide range in unit force despite a dramatic reduction in the size and range of muscle fibers.

The relative contribution of muscle fiber size, SF, and IR to determining the normal range of unit force has been controversial for many years (cf. Burke 1981; Mendell and Henneman 1981; Stein et al. 1991). In our experiments, the contribution of CSA was eliminated since all fibers within a single MU spanned the entire range of all fibers in the stimulated muscle. In addition, conversion of muscle fibers from FG and FOG to SO eliminated SF as a factor. In this way we showed that the remaining 35- fold range in unit force must be attributed to remaining differences in IR which is not altered by the chronic stimulation. This conclusion was supported by counting glycogen-depleted muscle fibers in normal muscles (Fig. 6.8).

Difference in mean CSA between the smallest S unit and the largest FF unit is only approximately 4- fold in the cat MG muscle as compared with the 100- fold difference in unit force (Fig. 6.3A). When differences in CSA between MUs is eliminated as a contributing factor to variations in unit force a 35-fold range in force remains. It leaves a 35- fold even when phenotype is not the same. The range in unit force was not different in muscles stimulated for 6 weeks compared with muscles stimulated for 20 weeks even though there were 3 fiber types present at 6 weeks and only SO fibers at 20 weeks (cf. Figs. 6.3B and 6.5A). These results suggest that SF in only moderately regulated between different fiber types in the cat MG muscle. This is consistent with direct measurements of SF from skinned cat MG muscle fibers which showed no significant differences between fast and slow muscle fiber types (Lucas et al. 1987). SF did not vary as a function of unit force in the rat TA muscle (Tötösy de Zepetnek et al. 1992) and only differed by 1.5- fold between S and FR units in the rat soleus (Chamberlain and Lewis) and by 1.4- fold between S and FF units in the cat TA (Bodine et al. 1987) muscles.

Taken together, these results indicate that SF is a relatively minor determinant in the overall range in unit force. The range in force must therefore be primarily due to differences in mean muscle fiber CSA and IR. Since the mean muscle fiber CSA within a single MU in stimulated muscles are not different, the remaining 35- fold range in force is primarily due to the wide range in IR. Similarly, in the cat soleus muscle, which has only S units and SO muscle fibers, the 13- fold variation in unit force (Henneman et al. 1965) can be attributed to a 13- fold range in IR. The range in unit force in stimulated MG muscles is approximately 2 times that of the cat soleus indicating that MUs in MG muscle have larger IRs.

Several studies, using indirect methods to determine IR, indicated that differences in SF, and not IR, is the primary factor determining the range in unit force (Burke and Tsairis 1973; Dum et al. 1982; Gordon et al. 1988). In these studies, the relative IR of each MU type was calculated to be the ratio of the proportion of muscle fibers classified histochemically as FG, FOG and SO and their corresponding physiological types (Burke and Tsairis 1973). Since IR is calculated as the ratio of 2 estimated values any error is estimating the relative proportion of muscle fiber or MU types will greatly over or underestimate their relative IR and overestimate the contribution of SF (see Discussion in Tötösy de Zepetnek et al., 1992). Estimating the relative IRs in a large heterogeneous muscle, such as the cat MG, is extremely difficult because the muscle is highly regionalized with respect to the different muscle fiber types (Chapter 3) making it difficult to accurately determine the true proportion of muscle fiber types from randomly sampling areas across the dorsal-ventral muscle boundaries. Our results in the chronically stimulated muscles, where we eliminated CSA and SF as contributing factors to the range in unit force, revealed that there is a very wide range of IRs which is the most important factor regulating unit force. The direct measurement of IR from glycogen-depleted MUs provide supporting evidence and is a reliable means of determining the relative contribution of IR even in the complex MG muscle. Once the pinnation angle and fiber length are taken into account the absolute values of IR, which is 2-3 times larger than the number of depleted muscle fibers counted in a single muscle cross-section, are valid (Chapter 4) even though viewed with extreme caution by Burke and Tsairis (1973).

The 30 to 35- fold range in IR found in cat MG muscle is much higher than the 3 and 6- fold ranges of IR in the rat TA (Tötösy de Zepetnek et al. 1992) and cat TA (Bodine et al. 1987) muscles, consistent with the larger 100- fold range in cat MG unit force compared with the 16 and 10- fold range in unit force reported in rat TA (Tötösy de Zepetnek et al. 1992) and cat TA (Bodine et al. 1987) muscles, respectively.

In comparison, the mean muscle fiber CSA varied over a 4.5- and a 2- fold range in the rat (Tötösy de Zepetnek et al. 1992) and cat TA muscles (Bodine et al. 1987), respectively. These results indicate that the relative contribution of muscle fiber CSA in the rat TA muscle, to the overall range in unit force, is approximately 2 times that in the cat TA muscle.

Since the range in unit force is predominantly determined by mean muscle fiber CSA and IR, and because CSA only varies over a 2 to 4.5- fold range, it follows that IR must vary over a larger range in muscles with a greater range in unit force. Consequently, the variation in the range in unit force between muscles is primarily due to differences in the range in IRs and not to fiber size or SF. In support of these findings the average IR tends to vary with the size of the muscle in humans (Feinstein et al., 1955).

In summary, the results from this study show that chronically stimulated MUs do not become homogeneous with respect to force or muscle fiber CSA within a single MU. The remaining wide range in unit force following stimulation is due to the large variation in unit IR which is an intrinsic property of MUs and does not change with altered activity. Since IR has been shown to vary systematically with unit force (Fig. 6.7.; see also Stein et al. 1990; Tötösy de Zepetnek et al. 1992) and several studies have indicated that unit force covaries with physiological measures of motoneuron soma size (Burke et al. 1973; Dum and Kennedy 1980; Fleshman et al. 1981) and axon diameter (Gordon and Stein, 1982; McPhedran et al. 1965), the results of this study provide further support that motoneurons branch and innervate muscle fibers in a size-dependent manner as originally proposed by Eccles and Sherrington (1930) and later by Henneman and Olson (1965).

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7. GENERAL DISCUSSION

The aim of this study was to address 3 fundamental questions central to motor unit physiology: 1) size dependent branching, 2) muscle fiber plasticity, and 3) neural control of muscle fiber properties. Our results suggest that the motoneuron size, or properties associated with size, determines the final number of muscle fibers that the motor axon supplies. Motoneurons branched in a size dependent manner in partially denervated muscles and in muscles reinnervated after nerve crush or complete transection and repair. However, both the dramatic alteration in the pattern of branching in reinnervated muscles and the restricted MU size in muscles reinnervated after N-M suture suggests that the pattern of branching of regenerating nerves is also highly dependent on the growth environment of the distal nerve stump and denervated muscle. Why muscle fibers within a single MU are consistently more clumped as compared to normal is not known. Clearly, the factors that promote motor axon branching during development are different from those during reinnervation.

Recent experiments by Landmesser and colleagues (1992) in the developing neuromuscular system of the chick embryo may provide some insight into resolving these differences. In their studies, they have shown that motoneurons in the slow and fast regions of developing chick iliofibularis muscles differ remarkably in their branching patterns. The extent of nerve branching in these two regions appears to be regulated by relative levels of expression of L1 and the degree of polysialiation of neural cell adhesion molecule (N-CAM) (Landmesser et al. 1990). L1 is a cell adhesion molecule which promotes axon-axon interaction (Lemmon et al. 1989; Rathjen et al. 1987;

Stallcup and Beasely 1985) while polysialiation of N-CAM is essential for branching and growth of axons across myotubes (Landmesser et al. 1988;1991). The extensive branching of nerves in the fast region of the iliofibularis muscle is believed to be due to high levels of polysialic acid (PSA) present on N-CAM which decrease cell adhesivity by inhibiting homophilic interactions between L1 molecules expressed on apposing nerves. Conversely, the small number of branches in the slow region is due to an absence of PSA on N-CAM which consequently promotes fasciculation by increasing contact between nerves and thereby permitting L1 mediated nerve-nerve adhesion (Landmesser et al. 1991).

Following complete nerve transection L1 is reexpressed along the transected peripheral nerves (Sanes and Covault 1985) while N-CAM is upregulated on the surface of denervated muscle fibers (Covault and Sanes 1985). After nerve section motor axons regenerate beyond the lesion site and randomly enter endoneurial tubes in the distal stump. Although transected axons rarely reinnervate their original muscle fibers (Sunderland 1978) the cell adhesive properties of the distal stump provide a favorable growth environment to guide the axon to the original synaptic site (Gutmann and Sanders 1943; Sanes and Covault 1985). It is conceivable that adhesion between regenerating axons is stronger than the signal from the muscle fibers which normally encourages nerve branching possibly because the level of PSA on N-CAM is low. If this is indeed the case, axons may be restricted in their capacity to divide at all of their original branching points. To compensate for the reduced number of proximal branches, the regenerating motoneurons extend many more terminal branches where the local stimuli from denervated muscle fibers promotes axon branching (Slack and Pockett 1981).

Whether or not the level of expression of PSA on regenerating nerves is a factor restricting proximal branching is not known. However, if the level of PSA is a factor, it may have important clinical applications since upregulating PSA on regenerating nerves may increase the extent of proximal branching within the muscle, which in turn will allow for larger MUs, particularly in smaller muscles.

Enlarged MUs in partially denervated and reinnervated muscles reestablished the normal range in contractile speed and the size relationship between twitch contraction time and unit tetanic force returned. In addition the mATPase staining intensities of muscle fibers within a single reinnervated MU was uniform. The results show that the contractile and muscle fiber properties of enlarged MUs are regulated by the reinnervated motoneuron and that there is a high degree of plasticity within reinnervated muscle fibers. However, several lines of evidence suggest that neural regulation of MU properties may be incomplete. Several reinnervated MUs elicited abnormal sag responses, and there was a substantial increase in the number of FI units while the cross-sectional areas of muscle fibers within a single MU spanned a broader range than normal. These results suggest a greater degree of heterogeneity within reinnervated MUs compared to normal.

Thus reinnervating motoneurons have a limited ability to control the size of muscle fibers and their metabolic properties. Incomplete transformation of the contractile and metabolic properties of reinnervated MUs is most striking in cat soleus muscles reinnervated by "fast" motoneurons (Chan et al. 1982; Dum et al. 1985;

Foehring et al. 1987; Gillespie et al. 1987). This observation implies that adult cat soleus muscles do not exhibit the same degree of phenotypic plasticity as muscle fibers in mixed muscles which, in turn, may reflect intrinsic properties of the muscle fibers which originated during development (Foehring et al. 1987; Gordon and Pattullo 1992). However, mechanical loading of muscle fibers can also alter their phenotype such that there is an increase in SO fibers in muscles immobilized in a stretched position (Pattullo et al. 1992). It may be possible that the mechanical loading of the soleus muscle induces SO fiber phenotypes and subsequently the muscle fibers appear more resistant to neural regulation. This hypothesis is readily testable by altering the normal mechanical load of the soleus muscle by cross-uniting its tendon with the tendons of the flexor muscles at the same time as cross-reinnervating the soleus with nerves to a fast-twitch muscle. If, under these conditions, the muscle fiber properties of the cross-reinnervated soleus is changed, then mechanical loading can be implemented as a factor that reduced the neural influence of the reinnervated motoneuron.

In summary, the neuromuscular nervous system displays a remarkable potential for recovery following nerve injuries. Regenerating motor axons retain the capacity to branch in a size dependent manner such that the normal order of motoneuron recruitment activates MUs from smallest to largest. However, these results show the importance of the growth environment in determining the extent of branching which in turn ultimately governs the size of the MU. A better understanding of the factors that promote or restrict branching during regeneration may lead to improved methods for treating peripheral nerve injuries.

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